Vasoactive intestinal peptide stimulates mucus secretion, but nitric oxide has no effect on mucus secretion in the ferret trachea

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Kim, Jung-Soo, Kosuke Okamoto, Shinobu Arima, and Bruce K. Rubin. Vasoactive intestinal peptide stimulates mucus secretion, but nitric oxide has no effect on mucus secretion in the ferret trachea. J Appl Physiol 101: 486-491, 2006. First published April 27, 2006; doi:10.1152/japplphysiol.01264.2005.—Vasoactive intestinal peptide (VIP) and nitric oxide (NO) are neurotransmitters involved in the regulation of bronchial and pulmonary vascular tone. Published studies of the effects of VIP on airway mucus secretion have yielded conflicting results. The purpose of this study was to determine the effect of VIP on mucus secretion in the ferret trachea and if this effect was influenced by NO. We used a sandwich enzyme-linked lectin assay to measure mucin secretion and a turbidimetric assay to measure lysozyme (serous cell) secretion from ferret tracheal segments. VIP (10^{-7} M) increased mucin secretion over 2 h. VIP (10^{-9} to 10^{-3} M) stimulated mucin secretion in a dose-dependent fashion. VIP-induced mucin secretion was partially blocked by a VIP receptor antagonist (a chimeric VIP-putative adenylate cyclase-activating peptide analog, VIP receptor agonist) at a 10-fold excess concentration. At all concentrations tested, neither NO^\text{nitro}-L-arginine methyl ester, an inhibitor of NO synthase, nor S-nitroso-N-acetyl-penicillamine, an NO donor, had any significant effect on constitutive or VIP-induced mucin secretion. We conclude that VIP-stimulated mucin and lysozyme secretion was both time dependent and dose dependent and that NO neither stimulates nor inhibits mucus secretion in the ferret trachea.

mucin; lysozyme; neuropeptides; nonadrenergic noncholinergic nervous system

VASOACTIVE INTESTINAL PEPTIDE (VIP) is the most abundant neuropeptide isolated from lung tissue and is a dilator of vascular and airway smooth muscle (2, 35). VIP has been localized to the airway submucosal glands of many species, including humans and ferrets (4, 8, 10, 22, 32). The density of VIP-positive nerves is significantly higher in the glands of bronchitic than in nonbronchitic subjects (23). Thus we speculated that VIP may contribute to the control of airway mucus secretion.

The neural control of mucus secretion in the airway has been very well reviewed by Rogers (25, 28). Published studies of the effects of VIP on mucus secretion have yielded conflicting results. VIP has been reported to stimulate mucous glycoconjugate release from ferret trachea (24), gland ecocytes in the ferret trachea (12), secretion from isolated feline tracheal submucosal glands (29), and isolated rat trachea (33). In contrast to these reports, VIP has been reported to inhibit constitutive and methacholine (MCh)-stimulated release of both mucous glycoconjugate and lysozyme in the human airway (6), inhibit mucus secretion from the ferret trachea (22, 34), and has no effect on secretion from feline tracheal explants (29).

Nitric oxide (NO) plays an integral role in many physiological events, including regulation of bronchial (15) and pulmonary vascular tone (3). VIP and NO synthase (NOS) are colocalized in ferret tracheal neurons (7, 10, 25), and NO is thought to be a critical mediator of signaling by neuropeptides such as VIP (3). VIP produces pulmonary vasodilation in a dose-dependent manner, and this effect has been reported to be inhibited by l-N-nitro-arginine, a NO inhibitor, but was restored by the addition of l-arginine, a substrate of NO (13). VIP increased saliva secretion during parasympathetic stimulation in the ferret and this was abolished by NO^\text{nitro}-L-arginine methyl ester (l-NAME), a NOS inhibitor (30). l-NAME is also reported to partially inhibit VIP-induced relaxation of guinea pig intestinal smooth muscle cells (27).

It has been reported that NO stimulates mucus secretion in rat gastric mucosal cells (5), but the role of NO in airway mucus secretion and, in particular, VIP-induced mucus secretion remains uncertain. One study demonstrated that NO acts as an endogenous inhibitor of mucus secretion in the ferret airway in vitro (12). In contrast to this, another study using guinea pig tracheal epithelial cells showed that l-NAME did not affect mucin secretion by itself but inhibited the stimulatory effect of inflammatory mediators, which significantly enhanced mucin release (1).

In this study, we evaluated the effect of VIP on mucus secretion and the role of NO and NO inhibitors on baseline and VIP-induced mucus secretion in the ferret airway.

METHODS

All of the chemicals and reagents used were purchased from Sigma (St. Louis, MO), unless otherwise specified. This study was approved by the Wake Forest University Animal Care and Use Committee.

Short-term organ culture. The ferret has often been used as a model to evaluate the effect of interventions on mucus secretion, especially from submucosal glands (16, 17, 28). Young adult male ferrets (~1,200–1,800 g body wt) were obtained from Marshall Farms (North Rose, NY). Ferrets were killed with an intraperitoneal injection of pentobarbital (120 mg/kg body wt), and the trachea from larynx to carina was immediately removed. Each trachea was divided into eight roughly equal segments from the cricoid cartilage to the carina. By using eight segments per trachea, we were able to assess baseline and stimulated secretion in each airway. The segments were weighed and then immersed in 7 ml of Krebs-Henseleit solution (KHS: 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 3.4 mM CaCl₂, 2 g/l D-glucose, pH 7.4) at ferret body temperature, 38°C. After a 2-h recovery, the KHS was changed, and the segments were incubated for 30 min with KHS alone (period 1). They were then incubated for 2 h in KHS + VIP (10^{-7} M) (control period 2). At the end of control period 2, samples were taken for mucin and lysozyme assays.

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incubated for another 30 min with test agents: MCh as a positive control, VIP, L-NAME with and without VIP, or KHS as a negative control (period 2).

The relative contribution by mucous and serous cells to the secretion was evaluated by measuring the amount of mucin glycoconjugates, a marker for mucous cell secretion (16, 17), and lysozyme as a marker of serous cell secretion (31). A secretory index (SI) expressing the relative increase in secretion under experimental conditions for each tracheal segment was calculated as the mucin or lysozyme concentration after 30 min exposure to the test agent divided by the concentration measured after 30 min exposure to KHS alone (period 1) in each segment. The relative change in the SI (RSI) was calculated using the SI of stimulated mucin or lysozyme secretion divided by that of unstimulated (KHS) secretion in each animal. This calculation made the RSI of the control group (KHS alone) equal to 1. The effects of agents were determined by comparing the RSI of the treated samples with those of matched control samples.

**Mucin analysis by sandwich enzyme-linked lectin assay.** Mucin is a complex secretion composed of water, mucin, ions, secreted proteins, phospholipids, etc. Mucin is secreted from mucous cells and glands and lysozyme from the serous glands. We have shown that, in the ferret, mucin can be used as a marker of mucous secretion and lysozyme as a marker of serous gland secretion. Ferret tracheal mucins have an abundance of galactose-N-acetylα-1–3 (fucose-α-1–2) galactose-R (20). These antigens can be detected by Dolichos biflorus agglutinin (DBA). This binds specifically to goblet cells and submucosal glands in the ferret trachea. DBA does not identify membrane bound mucins or mucin that has been deglycosylated (17).

A sandwich enzyme-linked lectin assay was used to measure DBA-associated mucous glycoconjugates (16). A 96-well microtiter plate was coated with 60 μl of DBA (8 μg/ml in PBS) and incubated at room temperature overnight. After rinsing four times with PBS with 0.05% Tween 20 (PBS-Tween), the plate was exposed to sample buffer and incubated at 37°C for 2 h. It was then incubated with 50 μl of DBA conjugated with horseradish peroxidase (0.25 μg/ml) in PBS containing 1% BSA. Before and after this step, the plate was washed four times with PBS-Tween. One hundred and fifty microliters of tetramethylbenzidine (0.42 mM) in citrate-acetate buffer (pH 6.0) were then added to each well and incubated for 10 min. The reaction was stopped by adding 50 μl of 4.7 N H2SO4. Color development was read as the difference in absorbance at 450 and 650 nm in an ELISA reader. The concentration of mucin was calculated by comparison with asialo bovine submaxillary mucin: 20–200 ng/ml. Concentration curves were also developed for type II porcine gastric mucin and were nearly identical.

**Lysozyme assay.** Lysozyme is a bacteriolytic enzyme found in airway fluid. Its only source in the ferret airway is reported to be the mucous cells of submucosal glands (31). The lysozyme concentration of the mucous samples was measured by a turbidimetric assay, which relies on the ability of lysozyme to break down the cell wall of the bacterium Micrococcus lysodeikticus. A 0.1-ml volume of sample was added to 0.9 ml of PBS at pH 6.0 containing M. lysodeikticus (0.3 mg/ml), sodium azide (1 mg/ml), and BSA (1 mg/ml). The reaction mixtures were then incubated in 24-well culture plates for 4 h at 37°C. After incubation, 300 μl of the reaction mixture were moved to a 96-well microtiter plate, and the optical density of each solution was measured at a wavelength of 450 nm in an ELISA reader. The standard curve was constructed by plotting the reduction in optical density against a known concentration of hen egg white lysozyme (1.6–100 ng/ml).

**Data analysis.** Statistical analysis of data was performed using the Stat View 5.0 statistics software (SAS Institute, Cary, NC). After verifying that data were normally distributed about the mean, these data were analyzed by ANOVA to assess secretory dose response and the effect of agents used. A probability of <0.05 was taken as significant. Results are presented as means ± SE.

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**RESULTS**

**Kinetics of VIP-induced mucin secretion.** Tracheal segments were incubated in KHS with VIP (10⁻⁸ to 10⁻⁶ M) or KHS alone, and the incubation solution was collected after 5, 10, 20, and 30 min. The amount of mucin released from control tissue and tissues exposed to VIP increased over a 30-min period (Fig. 1A). Over a 2-h period, mucin secretion continued to increase in response to 10⁻⁷ M VIP and 10⁻⁵ M MCh (Fig. 1B).

**Mucin and lysozyme secretion induced by VIP.** VIP (10⁻⁹ to 10⁻⁵ M) increased mucin (Fig. 2A) and lysozyme (Fig. 2B) secretion over control (RSI) in a dose-dependent fashion.

A chimeric VIP-pituitary adenylyl cyclase-activating peptide analog [VIP receptor antagonist (VRA) from Sigma] is reported to be a specific VRA with no reported cross-reactivity (11). We added 10⁻⁷ M and 10⁻⁶ M VRA to 10⁻⁷ M VIP and compared lysozyme and mucin secretion with that secreted in response to 10⁻⁷ M VIP or to KHS alone. VRA (10⁻⁷ M) had no effect on 10⁻⁷ M VIP-induced mucin secretion, but 10⁻⁶ M VRA decreased mucin (61.5%, Fig. 3A) and lysozyme (88.4%, Fig. 3B) secretion stimulated by 10⁻⁷ M VIP (P < 0.05 each). VRA had no direct effect on secretion.

**Effect of L-NAME (NOS inhibitor) on constitutive and VIP-induced mucin secretion.** VIP (10⁻⁶ M) significantly induced mucin and lysozyme secretion. L-NAME (10⁻⁶ to 10⁻⁴ M), an inhibitor of NOS, had no significant effect on constitutive or

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Fig. 1. Kinetics of vasoactive intestinal peptide (VIP)-induced mucin secretion. **A:** mucin secretion increases over 30 min from both control tissue and tissues exposed to VIP (10⁻⁸ to 10⁻⁶ M). **B:** mucin secretion increases over a 2-h period from both control tissue and tissue exposed to VIP (10⁻⁷ M) or methacholine (MCh; 10⁻⁵ M). KHS, Krebs-Henseleit solution. Data are expressed as means ± SE; N, no. of experiments. *Significant increase in secretion compared with control tissue (P < 0.05).
VIP-induced mucin or lysozyme secretion (Fig. 4, A for mucin and B for lysozyme). A 1-μmol concentration of VIP was chosen to ensure that there would be a large enough response to VIP to pick up a weaker signal of inhibition. This concentration of VIP has been used by other investigators evaluating the regulation of mucous secretion (12, 22).

**Effects of SNAP (NO donor) on constitutive and VIP-induced mucous secretion.** VIP (10^-9 to 10^-5 M) significantly increased mucin and lysozyme secretion over control, but SNAP (10^-6 to 10^-4 M), an NO donor, had no significant effect on constitutive or VIP-induced mucin or lysozyme secretion (Fig. 5, A for mucin and B for lysozyme).

**DISCUSSION**

We have shown that VIP stimulates mucin and lysozyme secretion in the ferret trachea in a dose-dependent manner, and VIP continued to induce secretion over a 2-h exposure period. A VRA at a 10-fold excess concentration partially blocked both mucin and lysozyme secretion. These data are similar to those of Fishbein and colleagues (11), who showed that this same antagonist had no effect on VIP-mediated pancreatic secretion in guinea pigs, but there was a 40% inhibition of secretion when administered in a 10-fold excess concentration. Inhibition of NO or NO donation had no significant effect on mucin secretion alone or in combination with VIP.

The reported effects of VIP on airway mucus secretion are conflicting. VIP has been reported to inhibit MCh-induced secretion in the ferret trachea (34). However, in that study, the total secretion volume was measured rather than measuring only mucin secretion. A common method to measure mucin secretion is to incubate cells or tissues with radioactive precursors of mucins and then to evaluate the release of radio-labeled mucin (6, 12, 22, 24, 29). Using this method, VIP has been reported to stimulate mucous glycoconjugate release from ferret trachea (24), gland exocytosis in the ferret trachea (12, 22), and mucin secretion from isolated feline tracheal submu-
VIP at 1 and 10 μM stimulated secretion from the ferret trachea in vitro, but VIP also inhibited cholinergic neural secretion (22). The stimulatory action of VIP alone on mucus output is thought to be compensated for by its inhibition of cholinergic stimulated mucus secretion (10).

Our laboratory has previously shown that DBA binds to blood group antigens and that, in the ferret trachea, this substantively recognizes only mucin (16), that mucin secretagogues will increase the secretion of mucin-like glycoconjugates, and that this induced secretion can be blocked by specific agonists of secretion (17). In the study reported here, we temporally measured VIP and MCh-stimulated mucin glycoconjugate secretion from 5 min to 2 h and found a constant increase in mucin secreted over the full 2 h. This suggests that VIP and MCh do not transiently increase mucin secretion. Therefore, we do not believe that differences in sampling times explain the difference in reported results, and, indeed, these results are consistent with studies that measured mucin secretion in response to VIP.

**Fig. 5.** Effect of S-nitroso-N-acetyl-penicillamine (SNAP) on constitutive and VIP-induced mucus secretion. VIP (10⁻⁶ M) significantly increased mucin and lysozyme secretion over control (KHS alone). SNAP (10⁻⁴ to 10⁻⁶ M), a nitric oxide donor, has no significant effect on constitutive or VIP-induced mucin (A) or lysozyme (B) secretion. Data are expressed as means ± SE. Numbers in parentheses represent the number of experiments. Significant increase over constitutive secretion: *P < 0.05 and **P < 0.01.

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VIP-stimulated mucin secretion is not inhibited by a mixture of tetradoxin, atropine, L-propranolol, and phenotolamine, suggesting that VIP may act on submucosal glands via specific VIP receptors (24). We have shown that the mucin secretagogue effect of VIP can be largely inhibited by a VRA, confirming that VIP-stimulated secretion is, at least in part, mediated by receptor stimulation.

There are few reported studies evaluating the effect of NO on airway mucus secretion. NO and VIP are thought to be released with cholinergic neurotransmitters and to regulate their effects (8). NO is reported to inhibit both basal and neurogenic stimulation of mucus secretion (26). One study using guinea pig tracheal epithelium showed that the stimulatory effect of inflammatory mediators, including histamine, platelet-activating factor, TNF-α, and xanthine oxidase, was inhibited by L-NAME but that 1-NAME alone did not directly affect mucin secretion (1). In contrast to this, it has also been reported that Nω-monomethyl-L-arginine, another inhibitor of NOS, increased basal output of macromolecules and that FK409, a NO donor, reduced basal secretion in the ferret trachea (26). In the rat, nasal perfusion with 1-NAME blocks albumin leakage but not mucin secretion in response to histamine (19). We found that neither 1-NAME nor SNAP had a significant effect on constitutive or VIP-induced mucus secretion. Explanations for the difference in the effect of NO on airway mucus secretion may be due to the species studied, incubation methods, or materials used (18, 28). Comparing the amount of mucin released from whole tissue before and after drug treatment can produce misleading results. By evaluating mucin secretion as the RSI (normalized to control cells), we eliminate variations due to differences in absolute mucin and lysozyme secretion in different size tissue sections.

The published literature regarding the effect of VIP on mucus secretion is contradictory. Although there are studies that suggest that VIP is a secretagogue, there are others that suggest that VIP is anti-inflammatory and inhibits mucus secretion. After demonstrating that VIP is a potent secretagogue of both mucin and lysozyme, in both a time- and dose-dependent fashion, we then evaluated if this was receptor and VIP-ligand mediated. In studies in which VIP was demonstrated to be secretagogue, some data suggested that this effect was receptor mediated and regulated by NO, whereas other studies suggested that this secretion is independent of NO. We confirmed that secretion induced by VIP was independent of NO and, as well, that NO did not appear to be a secretagogue in the ferret trachea. Finally, we wished to demonstrate that, despite the fact that VIP-induced secretion was not mediated by NO, secretion was nevertheless receptor mediated.

In experiments to investigate the effect of 1-NAME on the response to VIP, no pretreatment with 1-NAME was included before exposure to VIP. As a result, there is a possibility that the VIP could stimulate secretion before the concentration of 1-NAME inside the cell reached a level high enough to inhibit NOS. In Fig. 1A, this concentration of VIP begins to increase mucin secretion within 5 min, a time period that may be less time than it takes 1-NAME to inhibit NOS. However, at all concentrations tested from 10^{-6} to 10^{-3} M, neither SNAP nor 1-NAME used alone had any measurable effect on mucin or lysozyme secretion. This was true over the full observation period of 30 min while our studies showed a time-dependent increase in mucin secretion with 1 μmol VIP. Although this does not confirm a lack of involvement of NO, this lack of an effect with no VIP stimulation is supportive of this and consistent with previously published literature.

It was noted that there are significant differences in the size of the response to 1 μmol VIP in Figs. 2, 4, and 5. There are several possible explanations for this degree of variability. Tissues systems are intrinsically variable. We have shown differences not only between the response comparing different animals or on different days, but even at different levels of the trachea in the same animal (16, 17). The degree of variability comparing Fig. 2 with Figs. 4 and 5 is greater than we usually observe with this system, but, because the experiments were conducted by the same investigator under seemingly identical conditions, this variability is inexplicable.

We showed that VIP-induced secretion was not inhibited at equimolar concentrations by a specific antagonist, VRA, but was partially inhibited at a 10-fold excess of receptor antagonist with results nearly identical to previously reported studies of VIP-mediated pancreatic secretion (11). These results suggest that VIP stimulates mucus secretion in the ferret airway independently of NO but mediated, at least in part, by specific receptor activation. Furthermore, NO does not appear to play a role in constitutive or VIP-induced mucous glycoconjugate secretion in the ferret trachea. Finally, these data suggest that the secretion of mucin glycoconjugates from mucous cells and lysozyme from serous cells occurs together with VIP stimulation in the ferret trachea, an accepted model for submucosal gland secretion.

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