Aerosolization of P2Y2-receptor agonists enhances mucociliary clearance in sheep

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Aerosolization of P2Y2-receptor agonists enhances mucociliary clearance in sheep. J. Appl. Physiol. 87(6): 2191–2196, 1999.—The purpose of this study was to determine whether aerosolized INS316 (UTP) stimulates lung mucociliary clearance (MCC) in sheep and, if so, to compare its effects with INS365, a novel P2Y2-receptor agonist. In the first series of studies, we used a previously described roentgenographic technique to measure tracheal mucus velocity (TMV), an index of MCC, before and for 4 h after aerosolization of INS316 (10−1 M and 10−2 M) and INS365 (10−1 M and 10−2 M), or normal saline in a randomized crossover fashion (n = 6). In a second series of studies, we compared the ability of these agents to enhance total lung clearance. For these tests, the clearance of inhaled technetium-labeled human serum albumin (99mTc-HSA; Amersham Healthcare, Arlington Heights, IL) was measured serially over a 2-h period after aerosolization of 10−1 M concentration of each agent (n = 7). Aerosolization of both P2Y2-receptor agonists induced significant dose-related increases in TMV (P < 0.05) compared with saline. The greatest increase in TMV was observed between 15 and 30 min after drug treatment. The highest dose (10−1 M) of INS316 produced a greater overall stimulation of TMV than that of INS365 (10−1 M). Both compounds, compared with saline, induced a significant increase in MCC (P < 0.05) within 20 min of treatment. This enhancement in MCC began to plateau at 60 min. Although the response to INS316 started earlier, there was no significant difference between the clearance curves for the two compounds. We conclude that inhaled P2Y2-receptor agonists can increase lung MCC in sheep and that for P2Y2-receptor stimulation TMV accurately reflects changes in whole lung MCC.

The impairment of mucociliary clearance (MCC) in the airways is central to the pathophysiology of various respiratory disorders (28). The functional relationships among the ciliated surface epithelium, the mucous (gel) layer, and the underlying periciliary fluid (sol) layer constitute the basic interactions that promote mucociliary transport (9). Therefore, the disruption of any single component of this naturally occurring defense system can contribute to mucociliary dysfunction.

Exogenous nucleotides have been shown to modulate several physiological activities that are vital to the mucociliary apparatus. ATP and UTP have been shown to induce chloride secretion and water movement into the airway surface liquid (12, 14, 18), which hydrates mucus and optimizes periciliary fluid viscosity, both crucial to efficient ciliary beating (5). Other studies have demonstrated the direct stimulatory effects of ATP and UTP on ciliary beat frequency (29), as well as increased submucosal gland secretion (19) and goblet cell degranulation (13, 16). The combined effect of these activities should enhance MCC in the airways. Previous in vivo studies with ATP (15) and, more recently, UTP (20) confirm this hypothesis.

It is now recognized that the nucleotide-induced effects on the various components of the mucociliary system are mediated via specific extracellular receptors (P2 receptors) located in the airways (6, 7). The specific receptors in question have been designated P2Y2 receptors, based on their selectivity for the different nucleotides and their analogs (4, 25). Recent advances in receptor-mapping techniques have demonstrated that these receptors are highly expressed at the apical surface of airway epithelial cells (2). The direct stimulation of these P2Y2 receptors leads to a signaling cascade involving the activation of phospholipase C and eventual increase in cytosolic intracellular free calcium via increases in inositol 1,4,5-trisphosphate (3, 19, 21, 27). As mentioned above, this increased intracellular free calcium ultimately promotes a variety of functions within the airway epithelial cells (19, 27), which are now known to mediate MCC (20).

The purpose of the present study was to 1) determine whether INS316 (UTP) could stimulate lung MCC in sheep, 2) compare the effects of INS316 with a new P2Y2-receptor agonist INS365, and 3) compare the responses of tracheal mucus velocity (TMV), an indicator of whole lung clearance, with lung MCC, as measured by clearance of technetium-labeled human serum albumin (99mTc-HSA; Amersham Healthcare, Arlington Heights, IL) after stimulation with the above agonists.

METHODS

Agent preparation. All test compounds were obtained as dry powders from Inspire Pharmaceuticals (Durham, NC). On the day of the experiment, the amount of the test agent reflecting the molar concentration to be studied was dissolved in 7 ml of normal saline (0.9% NaCl). The solution was then filtered through a 0.22-µm nonpyrogenic filter (CoStar, Cambridge, MA), and 4 ml of this solution was used for aerosolization. These preparations contained no preservatives. Osmolarities of the low doses of INS316 (316 mosmol/l) and INS365 (330 mosmol/l) were similar to normal saline (308 mosmol/l).
However, the high dose ($10^{-1}$ M) of INS316 and INS365 had osmolarities of 547 and 580 mosmol/l, respectively.

Animal preparation. All procedures used in this study were approved by the Mount Sinai Animal Research Committee, which is responsible for ensuring the humane care and use of experimental animals. Adult ewes, 25–35 kg in weight, were restrained in an upright position in a specialized body harness adapted to a modified shopping cart. The animals’ heads were immobilized, and local anesthesia of the nasal passage was induced with 2% lidocaine. The animals were then nasally intubated with a 7.5-mm-ID endotracheal tube (ETT) (Mallinkrodt Medical, St. Louis, MO). The cuff of the ETT was placed just below the vocal cords, and its position was verified by a flexible bronchoscope. After intubation, the animals were allowed to equilibrate for a period of ~20 min before either TMV or MCC measurements began.

Measurement of TMV. TMV was measured by using a previously described in vivo roentgenographic technique (22). Eight to ten radiopaque Teflon disks, ~1 mm in diameter, 0.8 mm thick, and weighing between 1.5 and 2 mg, were introduced into the trachea via the ETT. The particles were insufflated by a catheter connected to a source of continuous compressed air generated at a flow rate of 3–4 l/min at 50 psi. The catheter remained within the ETT only briefly during actual insulation, and no contact with the tracheal surface was made. To minimize the possible impairment of TMV caused by the inflation of the ETT cuff (23), the cuff was deflated throughout the study except for the period of drug delivery. The cephalad-axial movements of the disks were then recorded by using videotaped fluoroscopy. Individual disk velocities were calculated by measuring the distance traveled by each disk over a 60-s period. A collar containing radiopaque markers of predetermined length was placed around the animal’s neck and was used as a standard to correct for magnification effects intrinsic to the fluoroscopy unit. The mean value of all the disk velocities was then calculated for that time point. New disks were insufflated at each time point. To avoid dehydration, the sheep were periodically gavaged with tap water via a nasogastric tube. The animals were weighed at each time point. To avoid dehydration, the sheep were periodically gavaged with tap water via a nasogastric tube. The animals were weighed at each time point.

Measurement of MCC. Aerosols of $^{99m}$Tc-HSA (3.1 mg/ml, ~20 mCi) were generated by a Raindrop Nebulizer (Nellcor Puritan-Bennett, Pleasanton, CA), which produces a droplet with a median aerodynamic diameter of 3.6 µm. The nebulizer was connected to a dosimetry system consisting of a saline nozzle and a source of compressed air (20 psi). The output of the nebulizer was directed into a plastic T piece, one end of which was connected to the sheep’s ETT and the other end to a piston respirator (Harvard Apparatus, South Natick, MA). The system was activated for 1 s at the onset of the respirator’s inspiratory cycle. The respirator was set at a tidal volume of 500 ml, an inspiratory-to-expiratory ratio of 1:1, and at a rate of 20 breaths/min to maximize central airway deposition. The sheep breathed the radiolabeled aerosol for 5 min. A gamma camera (Dyna Cam, Picker, Nothford, CT) was used to measure the clearance of $^{99m}$Tc-HSA from the airways. The gamma camera was positioned above the animal’s back, with the sheep in its natural upright position in the cart, so that the field of image was perpendicular to the animal’s spinal cord. External radiolabeled markers were placed on the sheep to facilitate proper alignment under the gamma camera. All deposition images where stored in a computer integrated with the gamma camera. A region of interest was traced over the image corresponding to the right lung of the sheep, and the counts were recorded. The counts were corrected for decay and expressed as percentage of radioactivity present in the initial baseline image. The left lung was excluded from analysis because its outlines are superimposed over the stomach, and counts can be affected by swallowed radiolabeled mucus.

PROTOCOL

TMV studies. All agents were studied in a randomized crossover fashion. The study solutions were aerosolized from a 4-ml volume via a Pari LC Jet Plus nebulizer (Pari Respiratory, Richmond, VA) to free-breathing sheep. The nebulizer was driven by compressed air with a flow rate of 8 l/min, which produces a droplet with a median aerodynamic diameter of ~5 µm. The time to deliver the solution was ~10–12 min. For TMV experiments, a baseline measurement was initially obtained, followed by aerosolization of either 0.9% normal saline (control), INS316 (at $10^{-1}$ M and $10^{-2}$ M), or INS365 (at $10^{-1}$ M and $10^{-2}$ M). TMV measurements were obtained immediately after, at 15 and 30 min after, and at 1, 2, and 4 h after agent administration. A washout period of at least 72 h separated studies with different agents. Agents were studied in a randomized crossover fashion.

To ensure that any effects of the high doses ($10^{-1}$ M) of compounds on TMV were not a result of nonspecific effects due to the increased osmolarity of these solutions, we measured TMV in three sheep before and serially after aerosolization of 1.8% wt/vol NaCl (616 mosmol/l).

MCC studies. For the MCC studies, a baseline deposition image was obtained immediately after radioaerosol administration. After acquisition of the baseline image, either 0.9% normal saline (control), INS316 ($10^{-1}$ M), or INS365 ($10^{-1}$ M) was aerosolized from a 4-ml volume by using the Pari LC Jet Plus nebulizer to free-breathing sheep. The nebulizer was driven by compressed air with a flow of 8 l/min. The time to deliver the solution was 10–12 min. On the completion of drug administration, the animal was immediately extubated. This was done to prevent false elevations in counts caused by aspiration of excess radiolabeled mucus from the ETT. Serial measurements of the radiolabeled material present in the lungs were obtained over a 2-h period at 5-min intervals for the first hour and then every 15 min for the next hour. A washout period of at least 7 days (half-life of $^{99m}$Tc = 6 h) separated studies with the different agents.

Statistics. Data were analyzed by using SYSTAT for Windows, version 5. TMV and MCC data were analyzed by using two-way repeated ANOVA (to assess overall effects), followed by a paired t-test to identify differences between specific pairs. Significance was accepted when $P = 0.05$. In addition, we compared the slopes of the mean MCC curves between 0 and 45 min using least squares linear regression analysis to determine whether there was a difference in the rapid clearance phases of the two compounds.

RESULTS

TMV. The TMV response curves for INS316 and INS365 are illustrated in Figs. 1 and 2, respectively. Aerosolization of both doses of these P2Y$_2$ receptor agonists produced immediate and significant increases in TMV over baseline. Aerosolization of 0.9% saline (control) did not affect TMV during this period (0–30 min). The maximum increase with saline at 15 min was only 4.2 ± 5.4% above baseline (100%). Overall, aerosolization of INS316 ($10^{-1}$ M and $10^{-2}$ M) significantly increased TMV over time ($P < 0.0001$) (Fig. 1). At $10^{-1}$ M, INS316 caused an immediate and
significant stimulatory effect on TMV (P < 0.05), which extended over the entire 4-h time course. The peak response (125.3 ± 6.5%) was seen 15 min after aerosol delivery (Fig. 1). At 10^{-2} M, INS316 did not exhibit a significant increase in TMV until 30 min after aerosol delivery (114.2 ± 4.1%) (Fig. 1). However, unlike the higher dose, aerosolization of 10^{-2} M INS316 sustained only a minimal increase in TMV response at 2 h (P < 0.1). By 4 h, TMV values for the lower dose of INS316 were comparable with the saline control. These temporal differences between the 10^{-1} M and 10^{-2} M doses of INS316 were not, however, statistically different (P = 0.640).

Similar to INS316, an overall significant increase in TMV was observed after aerosol delivery of INS365 (P < 0.0001; Fig. 2). At 10^{-1} M, INS365 produced an immediate and significant increase in TMV, which lasted for 1 h (P < 0.05). There was still some improvement at 2 h (P < 0.1) but smaller than that seen with the comparable dose (10^{-1} M) of INS316. The peak response with 10^{-1} M INS365 (144.3 ± 8.8%) was seen at 15 min after aerosol delivery. A comparable, but slightly less effective, response was seen with 10^{-2} M INS365. Comparisons of the two different INS365 doses at each time point revealed that the higher vs. lower dose produced a significantly larger response immediately after (P < 0.02) and 15 min after drug delivery (P < 0.04).

Although comparisons of the maximum peak increases in TMV (i.e., between 15 and 30 min after drug delivery) did not reveal any significant differences between the two compounds or doses (Fig. 3), analysis of the overall effect showed that INS316 at both 10^{-1} M (P < 0.0001) and 10^{-2} M (P < 0.02) simulated TMV more than the comparable doses of INS365.

To eliminate the possibility that the stimulatory effects of the 10^{-1} M doses of INS316 or INS365 on TMV resulted from an increased osmolarity rather than from P2Y2-receptor stimulation, we challenged sheep with an aerosolized solution of 1.8% NaCl (616 mosmol/l). Figure 4 shows that the high salt solution actually caused a more pronounced reduction in TMV over the 4-h period. Thus the stimulation in TMV seen with the high doses of INS316 or INS365 did not appear to result from the increased osmolarity.

MCC. MCC was expressed as the relative percent retention of inhaled 99mTc-HSA over time. Figures 5 and 6 illustrate the effects of INS316 and INS365, respectively, on MCC. With both compounds, MCC was significantly increased (P < 0.05) 20 min after aerosol dosing compared with saline, and then clearance of the 99mTc-HSA remained more rapid (with respect to saline) until ~50 min, when a plateau was observed. Although the response to INS316 occurred earlier than the response to INS365, when the two drugs were compared, there was no significant overall difference between the clearance curves of these two agonists.
Our results demonstrate that aerosolization of the P2Y2-receptor agonists INS316 and INS365 significantly increased TMV and whole lung mucus clearance in sheep airways. These findings confirm and extend previous findings in cats (15) and normal human subjects (20), which indicated that in vivo stimulation of P2Y2 receptors in the airways enhances mucus clearance. The peak stimulatory effect of these agents on TMV was seen between 15 and 30 min after aerosol delivery. This temporal feature in peak TMV response parallels previous observations by Wong and Yeates (29), who measured tracheal ciliary beat frequency in canines exposed to aerosolized ATP (10^{-5} M and 10^{-6} M) and GTP (10^{-5} M and 10^{-6} M). In their study, the maximal stimulatory effects occurred between 8 and 26 min after delivery, with ciliary beat frequency returning to baseline values by 30 min. Our findings demonstrated a more prolonged enhancement in TMV with both INS316 and INS365. The reasons for the increased response observed in the present study could include longer nebulization time (12–15 vs. 2 min) and higher concentrations of the agonists used or a decreased metabolism of the two compounds. We cannot, however, exclude the role of other factors that contribute to accelerating MCC, such as changes in mucous rheology and/or periciliary ionic concentrations.

Interestingly, the most prolonged increase in TMV was seen with the high dose of INS316. This was an unexpected finding, given that the in vitro studies of INS365 showed it to have more biological and chemical stability than INS316 (R. W. Dougherty, Inspire Pharmaceuticals, personal communication). Given these reports, the observation that INS316 had a more prolonged effect on TMV suggests that INS316 may be cleared more slowly than INS365 from the airways in vivo, and/or that P2Y2-receptor binding affinity for the INS316 was higher than that of INS365. Our data do not allow us to discern which mechanism(s) explains this in vitro-in vivo dichotomy, but our findings support the utility of in vivo studies to fully characterize the actions of novel pharmacological compounds.

That the change seen in TMV after aerosolization of the P2Y2 agonists results from receptor stimulation and not from nonspecific effects, mechanical effects related to experimental technique, and/or physicochemical properties of the inhaled aerosols is supported by two sets of control experiments with normal (0.9%) and hyperosmotic (1.8%) NaCl. If nonspecific mechanical effects were the cause of the increased TMV seen with these agents, then a similar increase would have been observed with the saline controls. Similarly, if the hyperosmolarity of the high doses of INS316 or INS365 were responsible for the increased TMV, then one would have expected a similar increase with 1.8% NaCl. The enhanced reduction in TMV over time in these 1.8% NaCl-treated animals supports the argument that INS316 and INS365 increased TMV via receptor stimulation.

Although these collective data provide strong support for the hypothesis of P2Y2-receptor stimulation, there is one caveat that needs to be mentioned. Transient decreases in systemic oxygen tension were noted...
after aerosolization of UTP in humans (20), and hypoxemia has been reported to influence various aspects of mucociliary function. Systemic hypoxemia was found to increase tracheal submucosal gland secretion in dogs (8), but, in two other studies, hypoxemia did not change ciliary beat frequency (10) or tracheal mucus transport (17). Oxygen saturation was not measured in this study, and there are no data in sheep examining the effects of systemic hypoxemia on mucociliary clearance. However, based on the variable data in other species, it is probably unlikely that, even if such changes in oxygen tension occurred in the present study, they would have influenced our results.

Because there are anatomical and functional differences between central and peripheral airways (11, 24), TMV, although a reliable marker of mucus clearance in the airways, may not precisely represent true whole lung mucus clearance. Therefore, we also measured whole lung MCC in this study. The results of the MCC curves confirm our TMV findings; i.e., both compounds significantly increased whole lung MCC. Furthermore, the improvement in clearance was seen between 15 and 20 min, which parallels the time of the peak increases in TMV. Our results combined with recent data demonstrating P2Y2-receptor presence throughout both central and peripheral airways (2) suggest that TMV is a reliable index for evaluating in vivo alterations in MCC by P2Y2-receptor agonists.

The stimulatory effect in MCC persisted for ~50 min, after which a plateau effect was observed and no further changes were measured. This plateau phenomenon may simply represent the effect of the initial clearance of the radioaerosol more centrally deposited in the tracheobronchial tree (1, 26) or retained radiolabel that cannot be removed by MCC. Additionally, factors such as the biometabolism of these agents, as well as their clearance by the bronchial circulation cannot be ruled out as possible contributing factors in producing this plateau effect.

Our finding that aerosolization of P2Y2-receptor agonists increases MCC corroborates the previous human clinical studies conducted by Olivier et al. (20), who demonstrated a 2.5-fold increase in MCC in normal volunteers after a dose of INS316. More recently, and of greater therapeutic implications, Shaffer and associates (25) demonstrated that aerosol delivery of INS316 increased MCC in patients with underlying pulmonary diseases specifically characterized by MCC dysfunction (i.e., in smokers and chronic bronchitis). Thus P2Y2-receptor agonists could represent a unique approach to enhancing impaired MCC associated with airway disease.

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REFERENCES


