**In Vitro Evaluation of the Ciliary Beat Frequency of the Rat Nasal Epithelium Using a High-Speed Digital Imaging System**

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**Mucociliary clearance (MC) is an important factor in determining nasal drug absorption and the ciliary beat of ciliated epithelial cells of the nasal mucosa is the driving force of MC. However, the relationship between MC and ciliary beat frequency (CBF) is still ambiguous. The purpose of this study was to establish an evaluation method of CBF as an index of mucociliary function and examine the relationship between MC and CBF. A sequence of images of ciliary beating of an excised rat nasal septum was captured using a high-speed digital video camera. CBF (beats per second, Hz) was determined from periodic changes in the contrast value of a specific location in a sequence of images. CBF under control conditions was 8.49±0.38 Hz, which is similar to values reported for cultured human nasal epithelial cells and rat tracheal cells. β-adrenergic and cholinergic antagonists decreased CBF, while β-adrenergic agonists and acetylcholine increased CBF. These results were similar with those observed for MC in our previous study. It was found that CBFs were significantly and linearly correlated with MC, indicating that MC is directly regulated by CBF and that this evaluation system allows the quantitative determination of nasal mucociliary function.**

**Key words** mucociliary clearance; ciliary beat frequency; high-speed digital imaging method; nasal absorption; cilia; nasal cavity

Nasal drug delivery has several advantages over oral drug administration. After the nasal application of drugs, a faster onset of action is expected due to no time being needed to reach the absorption site, a relatively large absorptive surface area, and high vascularization. Furthermore, it has been shown that bioavailability is also greater due to the avoidance of presystemic metabolism and/or chemical degradation.1,2 Therefore, the nasal route has been attractive as an alternative to oral administration, and there has been an increase in the number of drugs for nasal administration being reported over the last two decades.

Drugs nasally applied are known to be cleared from the nasal cavity by mucociliary clearance (MC), which is the translocation of mucus covering the nasal epithelium toward the pharynx. Therefore, the nasal absorption of drugs could be highly influenced by MC.1,2 However, few studies have been performed on the detailed relationship between MC function and nasal drug absorption. In a previous study, we established an in vitro MC evaluation system allowed us to quantitatively determine MC and estimate the effects of drugs, pharmaceutical additives, and physicochemical properties of nasal formulations on MC under physiological conditions using an excised rat nasal mucosa.3

Since MC is created by ciliary beats, ciliary beat frequency (CBF) is usually measured as an index of ciliary activity. Moreover, CBF has been utilized as an index of ciliary activity to evaluate the ciliotoxicity of nasal formulations.4,7 Some in vitro evaluation systems on CBF, utilizing a video, photoelectric detection, or high-speed digital imaging system, have been reported,2 where various types of cells, such as primary-cultured epithelial cells8–10 and single ciliary cells,11) were used for the evaluation of CBF. However, the relationship between MC and CBF is still quantitatively ambiguous12 and there has been no study on the CBF of the rat nasal epithelium and its relationship with MC. In the present study, we attempted to establish an evaluation system of CBF using the rat nasal mucosa and quantitatively estimate the relationship between MC and CBF.

**MATERIALS AND METHODS**

**Materials** Salbutamol (SBM) and terbutaline (TBL) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Hanks balanced salt solution (HBSS, pH 7.4) and urethane (ethyl carbamate) were supplied from Life Technology Corp. (Carlsbad, CA, U.S.A.) and Tokyo Chemical Industry Co. (Tokyo, Japan), respectively. Propranolol (PPl), atenolol (ATl), acetylcholine (ACH), atropine (ATRP), and cefazolin (CFZ) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Male Wistar rats were supplied from Shimizu Laboratory Supply Inc. (Kyoto, Japan).

**High-Speed Digital Imaging System** Photographs of beating cilia were taken serially with a high-speed digital video camera (IPX-VGA210-L, IMPERX, Boca Raton, FL, U.S.A.), equipped with a phase-contrast microscope (CKX41, Olympus, Tokyo, Japan). A sequence of images of cilia was captured for approximately 1 s at a rate of 100 frames per second and was recorded by Windows PC. The application software, EpiX Xcap-Ltd. (Arigo Corporation, Osaka, Japan) was used for the capture and analysis of images.

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CBF Evaluation  Male Wistar rats weighing 220–300 g were used in all animal experiments. All animal studies were previously approved by the Committee of the Animal Care of Shujitsu University and were conducted under the Guidelines in accordance with the Principles of Laboratory Animal Care (NIH Publication #85-23). Under urethane anesthesia, a section of the nasal mucosa was excised from the nasal septum and immersed in a dish containing HBSS at 37°C just after the excision. The dish was set on the phase-contrast microscope, and a sequence of images was recorded with the high-speed video camera.

Effects of Various Drugs on CBF  Drugs with four different modes of pharmacological action were used in this study. PPl and ATL, β-adrenergic antagonists, SBM and TBL, β-adrenergic agonists, ACH, a cholinergic agonist, and ATRP, an anticholinergic agent, were used to investigate their effects on CBF. CFZ, an antibiotic known as a first generation cephalosporin, was employed as a negative control. The section of the mucosa was immersed in HBSS just after excision. Serial images at three different locations on each mucosa were captured and CBFs were determined at three different points on each captured image(s). A value of CBF for a single isolated mucosa was calculated as the average of these values. Each study was carried out by using at least three to five different mucosae. After measuring the initial value, HBSS was replaced with that including a drug and serial images on each tissue preparation were captured every 90 s until 360 s.

Statistical Analysis  Results are expressed as the mean±S.E. Statistical significance of differences in the means was determined by Dunnett’s test by utilizing the statistical software, JMP® (SAS Institute Japan, Tokyo, Japan). Statistical significance of the correlation between CBF and MC was examined by Pearson’s method.

RESULTS

Determination of CBF  Figure 1 shows a typical example of a series of captured images. The image was taken approximately every 0.01 s. Although each cilium is not clear in the image, the photographs show that cilia are regularly beating back and forth. Since a periodically moving cilium in the region of interest obstructs the light, the contrast in the region of interest changes periodically as shown in Fig. 2A where contrast values at a pixel were plotted against the image number. Therefore, it was assumed that periodic changes in the contrast value were based on the periodic beating of cilia and that the frequency of the contrast was the same as CBF. Therefore, the CBF value was evaluated as the frequency of periodic changes in the contrast based on the time profile, and CBF was represented as beats per second (Hz). The CBF was determined by following the method as schematically described in Fig. 2B. In brief, the integer number (n) of cycle of waveform during the time of image capture, about 1 s, was divided by the time from start of the first cycle to the end of the final cycle (t) (CBF=n/t).

Figure 3 shows the time profile of CBF under control conditions, indicating that CBF did not change significantly during the experiment period, and that the CBF value under control conditions was 8.49±0.38 Hz.

Effects of Various Drugs on CBF  PPL or ATL tended to decrease CBF just after treatment with the drug solution at a low concentration (1 µM) and high concentration (100 µM) (Figs. 4A, B). CBFs during the 360-s exposure were significantly decreased by 25% with the low concentration and by 20% with the high concentration (Fig. 4C), but the effects of the β-adrenergic antagonists on CBF were unlikely to be dependent on concentration.

On the other hand, as shown in Fig. 5, treatment with β-adrenergic agonists tended to increase CBF. SBM signifi-
significantly increased CBF after a 90-s incubation regardless of the concentration and maintained a larger CBF than that of the control (Fig. 5A). After treatment with TB l, CBF at each time point also tended to increase (Fig. 5B) and the mean value during a 360-s exposure to 1 m was significantly larger than that of the control (Fig. 5C). There was no clear concentration dependency in the stimulatory effects of the β-adrenergic agonists on CBF.

ACH also tended to increase CBF. Particularly, a 90-s incubation with 100 µm ACH significantly increased CBF (Fig. 6A) and a 360-s exposure resulted in increases in CBF by 15 and 25% for 1 µm and 100 µm, respectively (Fig. 6C). In contrast, treatment with ATRP showed a tendency to decrease CBF and decreases in CBF of 22% and 18% were observed for 1 and 100 µm, respectively (Figs. 6B, C). These results suggest that cholinergic drugs would stimulate ciliary function, while anticholinergic drugs would suppress it.

The addition of CFZ did not cause any significant change in CBF at concentrations of 1 µm and 100 µm (Fig. 7), suggesting that CFZ does not affect ciliary function at these concentrations. No effect of CFZ on MC was also confirmed by following the method reported previously 3) (Table 1).

The relationship between CBF and MC was investigated utilizing our previously reported results for MC. In our previous study, the effects of the drugs used in the present study on MC were examined by a new in vitro MC evaluation system, where the transport rate of fluorescence microspheres (V_FMS) applied on the surface of the rat nasal mucosa was regarded as MC.5) Table 1 summarizes the MC and CBF values obtained in the present study. As shown in Fig. 8, CBF was significantly correlated with MC (R=0.81, p<0.001), which strongly indicates that MC is directly connected to and regulated by the ciliary beating of the nasal epithelium.

**DISCUSSION**

In the present study, images of the beating cilia of the excised rat nasal mucosa were taken by a high-speed digital video camera to evaluate the CBF of the rat nasal mucosa. To date, several studies on CBF have been reported, where a few types of cells from several animal species were employed to detect ciliary beating by a video, photoelectric detection, or high-speed digital imaging system.2) However, no study has been found on the CBF of the rat nasal mucosa. Since rats are often used as a model animal to study nasal drug absorption, it is important to investigate the relationship of drug absorption after nasal application with mucociliary function using nasal epithelia from rats.

By analyzing periodical changes in the contrast of images, the CBF of rat nasal epithelia under control conditions was determined to be 8.49±0.38 Hz. The average CBF of human nasal cells was reported to be 10 Hz, ranging from 8–11 Hz at temperatures between 20 and 45°C.14) Dimova et al. reported that the CBF of primary human nasal epithelial cells was 7.9±1.1 Hz (between 5.1 to 10 Hz).5) Hayashi et al. also reported that the CBFs of rat tracheal and distal airway cells using a slice preparation were 9.2±0.2 Hz and 9.0±0.2 Hz (range between 4 to 12 Hz), respectively.13) These reported values are similar to our results, suggesting that the evaluation system developed in the present study would provide us with an acceptable value for CBF and allow us to estimate the ciliary function of the rat nasal mucosa.
Fig. 4. Effect of β-Adrenergic Antagonists on the CBF of the Rat Nasal Mucosa

(A) Changes in CBF as a function of exposure time with the propranolol solution (○; 1 µM, ●; 100 µM). (B) Changes in CBF as a function of exposure time with the atenolol solution (○; 1 µM, ●; 100 µM). (C) Effect of β-adrenergic antagonists on CBF of the rat nasal mucosa. Each value is the average of a 90 to 360 s drug exposure (□; 1 µM, ■; 100 µM). *p<0.05, significantly different from control conditions. Data are expressed as the mean±S.E. of 3–5 independent experiments.

Fig. 5. Effect of β-Adrenergic Agonists on the CBF of the Rat Nasal Mucosa

(A) Changes in CBF as a function of exposure time with the salbutamol solution (○; 10 µM, ●; 1 mM). (B) Changes in CBF as a function of exposure time with the terbutaline solution (○; 10 µM, ●; 1 mM). (C) Effect of β-adrenergic agonists on CBF of the rat nasal mucosa. Each value is the average of a 90 to 360 s drug exposure (□; 10 µM, ■; 1 mM). **p<0.01, *p<0.05, significantly different from control conditions. Data are expressed as the mean±S.E. of 3–5 independent experiments.
To validate the \textit{in vitro} CBF evaluation system and examine the relationship between CBF and MC, drugs with four different modes of pharmacological action, which are known to have an effect on ciliary function\textsuperscript{15} and MC,\textsuperscript{3} were used to change mucociliary function in the present study. Drug concentrations used in this study were selected based on previous \textit{in vitro} study\textsuperscript{13,15,16} except for PPL and ATL. Because of no report of \textit{in vitro} study about the effect of $\beta$-adrenergic antagonists (PPL and ATL) on ciliary function,\textsuperscript{15} the concentration of these drugs were determined by previous report of \textit{in vivo} study.\textsuperscript{17} As shown in Figs. 4 and 5, treatment with $\beta$-adrenergic antagonists (PPL and ATL) or $\beta$-adrenergic agonists (SBM and TBL) decreased or increased CBF, respectively, suggesting that ciliary beating would be regulated through adrenergic receptors. It is known that the superficial epithelium expresses $\beta$-adrenergic receptors ($\beta$-ARs)\textsuperscript{18,19} and that $\beta$-adrenergic drugs affect ciliated cells through $\beta$-ARs.\textsuperscript{20} Many investigators have extensively studied the regulation of CBF using ciliated epithelial cells and have clarified that $\beta$-adrenergic agonists stimulate the CBF of ciliated epithelial cells.\textsuperscript{21} $\beta$-Adrenergic agonists have been shown to have various effects on epithelial cells, such as the stimulation of CBF\textsuperscript{21,22} and chloride secretion toward the airway lumen,\textsuperscript{23} which could contribute to the enhanced mucociliary function by $\beta$-adrenergic agonists. It has been reported that $\beta$-AR activation in ciliated cells increased CBF through the fol-
to 110.3 ± 2.3% and 119.5 ± 6.9%, respectively (Fig. 5C). These results are similar to the results reported previously, indicating that our in vitro system would be valid to evaluate the CBF of the rat nasal mucosa. We have already reported that SBM (10 μM) and TBL (10 μM) increased MC to 115% and 140%, respectively, although these effects were not concentration-dependent (Table 1). These results are also similar to the changes observed in the present study in CBF by SBM and TBL. In the case of β-adrenergic antagonists, Pavia et al. reported that PPL and ATL decreased tracheobronchial mucociliary clearance in healthy human subjects. In the present study, 1 μM and 100 μM of PPL and ATL significantly decreased CBF by 18–27% (Fig. 4), clearly indicating that β-adrenergic antagonists exerted an effect opposite to that of β-adrenergic agonists on the ciliary beating of the rat nasal mucosa. In our previous study, PPL and ATL significantly decreased MC in a dose-dependent manner (Table 1), suggesting that β-adrenergic antagonists would suppress MC by inhibiting the ciliary beating of the nasal mucosa, although their effects on CBF were not concentration-dependent (Fig. 4). Since very little research has been undertaken regarding the effect of β-adrenergic antagonists on ciliary function, further studies are needed to clarify the reason why the concentration-dependency was not observed for their effects on CBF.

ACH is a major regulator of airway epithelial functions such as mucus secretion, CBF, cell proliferation, and the release of CT, granulocyte macrophage colony stimulating factor (GM-CSF), and interleukin (IL)-8 to the airway surface. In general, ACH increases mucus secretion and stimulates CBF. Hayashi et al. also reported that ACH (100 μM) increased the CBF of tracheal ciliary cells by 23 ± 2% (Fig. 4). We confirmed that 1 μM and 100 μM ACH increased the CBF of the rat nasal mucosa by 15.8 ± 1.4% and 25.6 ± 13.2%, respectively (Fig. 6). On the other hand, anticholinergic agents are well known to inhibit mucociliary function. Groth et al. reported that ATRP delayed the mucociliary transport of mucus on the lung airway. It was also reported that ATRP not only delayed mucociliary transport, but also reduced CBF in the isolated airways of the rat, cat, hamster, and monkey, where ATRP caused a dose-related reduction in CBF, ranging from 8% at 10−6 g/mL (3.5 μM) to 31% at 10−3 g/mL (3.5 mM).

Table 1. Effect of Various Drugs on CBF and Mucociliary Clearance (MC)

| Drugs         | CBF (% of control) | MC (mm/min) | MC (% of control) |
|---------------|---------------------|-------------|-------------------|
| Control       | 100.0 ± 1.8         | 1.22 ± 0.20 | 100.0 ± 16.2      |
| Propranolol   | 80.0 ± 5.3          | 0.76 ± 0.25 | 61.9 ± 4.2        |
| Salbutamol    | 120.4 ± 4.1         | 1.41 ± 0.22 | 115.0 ± 17.0      |
| Terbutaline   | 119.4 ± 8.3         | 1.18 ± 0.16 | 96.4 ± 12.7       |
| Acetycholine  | 115.8 ± 1.4         | 0.95 ± 0.04 | 155.4 ± 17.2      |
| Atropine      | 82.5 ± 2.6          | 0.53 ± 0.06 | 43.6 ± 4.9        |
| Cefazolin     | 104.3 ± 1.0         | 1.25 ± 0.06 | 102.0 ± 5.0       |
|               | 96.5 ± 5.5          | 1.03 ± 0.05 | 84.6 ± 4.4        |

a) Results obtained in our previous study on MC. ** p<0.01, * p<0.05, significantly different from control conditions. Data are expressed as the mean ± S.E. of 3–5 independent experiments.

Fig. 8. The Correlation between CBF and Mucociliary Clearance (MC)
study also showed that ATRP decreased CBF to 78 and 82% of the control at concentrations of 1 µM and 100 µM, respectively (Fig. 6). As for the effects of ACH and ATRP on MC, 100 µM of ACH increased MC to 155%, while 100 µM of ATRP significantly decreased MC to 44% (Table 1). These results suggest that the effects of these drugs on MC and CBF are comparable and that ACH and anticholinergic agent (ATRP) show cilio-stimulating and cilio-inhibitory effects on the rat nasal mucosa, respectively. On the other hand, unexpectedly, these drugs failed to show a concentration-dependent effect on CBF (Fig. 6C). Since the nasal mucosa was directly immersed in the drug solution, the effects of these drugs on CBF may be saturated at the drug concentrations examined, suggesting that much lower drug concentrations should be examined in a future study.

CFZ is an antibiotic known as a first generation cephalosporin and was used as a negative control in the present study. Treatment with CFZ at 1 µM and 100 µM had no effect on CBF (Fig. 7) or MC (Table 1), indicating that CFZ has no effect on mucociliary function at the concentrations examined.

As described above, the results obtained for CBF in the present study are generally consistent with those for MC in our previous research. Finally, the relationship between CBF and MC was then examined and the relationship between the two parameters was found to be significant and linear (R^2 = 0.81, p < 0.001) (Fig. 8). This finding suggests that ciliary beating is indeed a driving force for MC and its speed would regulate the function of the nasal mucosa for drug clearance under the conditions examined in the present study, and that the in vitro CBF evaluation system is useful to quantitatively determine CBF and changes in it by drugs and pharmaceutical additives. On the other hand, the slope of the equation expressing the relationship between MC and CBF was 1.62, indicating that MC was affected more than CBF by the drugs examined. Since MC is caused by the interaction between ciliary movement and the mucus layer covering the mucosal surface, changes in MC may be amplified more than those in CBF. Furthermore, since the value of the y-axis of the equation was negative (~75.1), it was recognized that CBF may be needed over a given level to cause MC. These findings indicate that clarifying the relationship between MC and CBF should be necessary in order to evaluate the effects of drugs on mucociliary function, and that our present results should be useful in the quantitative estimation of mucociliary function based on results obtained for CBF.

CONCLUSION

The in vitro CBF evaluation system developed provided 8.49 ± 0.38 Hz as the CBF of the rat nasal mucosa, similar with those reported previously, and also indicated the effects of adrenergic or cholinergic drugs on CBF, consistent with their effects on MC. CBFs were significantly and linearly correlated with MC, indicating that ciliary beating is indeed a driving force for MC and that MC is regulated by ciliary beating. The in vitro evaluation system would help us to understand nasal mucociliary function, and the relationship between MC and CBF obtained in the present study would be useful in evaluating the changes in mucociliary function and their relationship with drug absorption after nasal application.
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