Aging causes a slowing in ciliary beat frequency, mediated by PKCe


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FOR PEOPLE 65 AND OLDER, pneumonia is the seventh leading cause of death (12) and the third most common reason for hospitalization (18). The elderly are four times more likely to develop pneumonia than younger adults (9), and nearly 90% of deaths due to pneumonia occur in those 65 or older (6). People aged 85 and older have the highest risk of pneumonia, with a nearly three times greater risk than those aged 65–69 (8).

Despite appropriate antibiotic therapy, the mortality of pneumonia in the elderly is higher than in younger populations (17). In fact, advancing age is independently associated with mortality (15). Little is known at the molecular level about how normal aging leads to an increased susceptibility to pneumonia and higher mortality.

The lung is continuously exposed to the outside environment, and has a complex innate immune system to defend itself from microbial infection. The first line of defense is mucociliary clearance. The conducting airways of the lung are lined with ciliated airway epithelium. When foreign particles or microorganisms try to invade the airway epithelium, they are trapped in the mucus layer, and the cilia beat in a coordinated manner to remove the invading pathogens. Ciliary beating in the nasal epithelium slows with normal aging in humans (1, 7). Surprisingly, ciliary beat frequency (CBF) of the lower airways in aging has not been measured in humans. However, the decrease in nasal CBF correlates with decreases in pulmonary clearance in humans (24), and the mechanism for this slowing is unknown. We have demonstrated lower airway ciliary slowing in a mouse model of aging that allows us to determine the mechanisms of ciliary slowing.

Although much is known about the mechanisms of stimulation of CBF, very little is known about the mechanisms of slowing baseline beating. We have recently shown that activation of protein kinase C epsilon (PKCe) plays an important role in ciliary slowing in a model of cigarette smoke and alcohol exposure (28), and in Haemophilus influenzae exposure (2). We hypothesized that in normal aging, ciliary slowing is mediated by PKCe.

METHODS

Mouse model. All experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center.

C57BL/6 mice aged 2, 12, and 24 mo and BALB/c mice aged 2, 5, 12, and 24 mo were obtained from the National Institute on Aging (Bethesda, MD) rodent colony. They were acclimated for 1 wk after arrival. The mice were euthanized by decapitation. The trachea and lungs were removed on bloc. The tracheas were separated and placed in DMEM. Tracheal rings were cut to a 0.5-mm thickness from the distal trachea for measurement of CBF. The rings were maintained in DMEM media at 37°C, 5% carbon dioxide overnight to equilibrate, then CBF was measured. The remaining tracheas were flash-frozen in liquid nitrogen and stored at −80°C until measurement of PKC isoform activity could be performed.

Measurement of CBF. CBF was measured using Sisson-Ammons video analysis (SAVA), lung; innate immunity; β2 agonists; DCP-LA

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were stimulated with the beta-agonist procaterol (100 nM) for 5 min, and CBF was measured again.

Measurement of motile cilia. In addition to CBF, SAVA was used to determine the number of motile cilia as previously described (23). Briefly, the number of motile points from each digital video image was determined using a software algorithm in SAVA. The algorithm assesses whether a change in light intensity occurs in a 4×4 pixel area. If there is a change in light intensity, those cilia are motile. For each 640×480 pixel video image, the number of motile points is calculated from a possible 19,200 total points. The entire lumen of each tracheal ring was used to measure CBF, and motile points were used to rule out selection bias. Each whole-field analysis was averaged for the total number of motile points.

PKCe activity assay. PKCe and PKCε kinase activities were measured from the tracheas of the BALB/c mice as previously described (29). Briefly, the tracheas were homogenized in lysis buffer, sonicated, and centrifuged at 10,000 g for 30 min at 4°C. The supernatant was removed (cytosolic fraction) and the pellet was resuspended in cell lysis buffer containing 0.01% Triton X-100 and sonicated again (particulate fraction). To measure specific PKCe isoform activity, 24 μg/ml phorbol 12-myristate 13-acetate (PMA; 30 mM dithiothreitol, 150 mM ATP, 45 mM Mg-acetate, PKC isoform-specific substrate peptide, and 10 μCi/ml [c-32P]-ATP were mixed in a Tris-HCl buffer (pH 7.5). Chilled (4°C) cell lysates (cytosolic or particulate) were added to 40 μl of the reaction mix and incubated for 15 min at 30°C. This mixture (60 μl) was then spotted onto P-81 phosphocellulose papers (Whatman, Clifton, NJ) halting the incubations. Papers were washed five times for 5 min in phosphoric acid (75 mM), washed in 100% ethanol for 1 min, dried, and counted in a scintillation counter (National Diagnostics, Atlanta, GA). PKC activity was expressed in relation to the total amount of cellular protein assayed as picomoles of phosphate incorporated per minute per milligram.

RNA extraction and real-time PCR. Tracheas from BALB/c mice aged 2, 12, and 24 mo were removed and stored in RNAlater (Sigma-Aldrich, St. Louis, MO). RNA was extracted using the MagMax kit (Applied Biosystems, Carlsbad, CA) as previously described (3). Briefly, the tracheas were homogenized in lysis binding solution (provided in the kit), with 2-mercaptoethanol (100 mM), and RNA was extracted following the manufacturer’s instructions. RNA quantity and quality were evaluated using spectrophotometry (Nanodrop, Wilmington, DE). Only samples that had a 260/280 ratio of >1.8 were used. Total cDNA was synthesized using the Taqman real-time PCR kit (Applied Biosystems) with 100 ng of RNA template and random hexamers according to the manufacturer’s directions. Real-time PCR was performed using a cocktail of 1× Taqman universal master mix (Applied Biosystems) and 1× primer/probe mix for PKRCE (PKCeε) (Mm 00440894; Applied Biosystems). Reactions were performed in duplicate. The plate was then placed in an ABI Prism 7500 sequence detection system (Applied Biosystems). Reactions underwent 2 min at 50°C, 10 min at 95°C, then 40 cycles of 15 s at 95°C, and 1 min at 60°C. Ribosomal RNA (Applied Biosystems) was used as an endogenous control. For relative comparison of PKCe to ribosomal RNA, we analyzed the cycle threshold value (Ct) using the ΔΔCt method (16) and reported the data as Ct normalized to ribosomal RNA.

PKCe ELISA. Total PKCe protein was measured from the whole lung homogenate of the C57BL/6 mice aged 2, 12, and 24 mo. One lobe of the lung was homogenized in 250 μl of phosphate buffered saline (PBS) using the Bullet Blender (Next Advance, Averrill Park, NY). The solution was sonicated, particles were removed by centrifugation, and the resulting supernatant was diluted 1:32. The quantity of PKCe protein was measured using a PKCe ELISA kit (USCN Life Science, Houston, TX) following the manufacturer’s directions. The concentration of PKCe protein was normalized to the total quantity of protein in the sample, and reported as nanograms PKCe per milligram of total protein.

Statistical analysis. Data are presented as means ± SE. GraphPad (La Jolla, CA) Prism software was used to perform one-way ANOVA with a Tukey’s posttest. Changes among the groups with P < 0.05 were considered statistically significant.

RESULTS

Aged mice have significant ciliary slowing at baseline. The baseline CBF in tracheal rings in BALB/c mice aged 2 mo was 16.41 ± 0.47 Hz (n = 15); in mice aged 5 mo it was 18.05 ± 1.58 Hz (n = 8) (Fig. 1). CBF was significantly slowed in the middle-aged mice (12 mo) at 14.49 ± 0.34 Hz (n = 12) (Fig. 1A). The aged mice (24 mo) were even slower, at 13.27 ± 0.68 Hz (n = 8) (Fig. 1A). The difference in CBF was statistically significant between the 2- and 12-mo-old (P < 0.05) and 2- and 24-mo-old mice (P < 0.001). The differences between the mice at 12 and 24 mo were not significant (Fig. 1A).

To ensure that ciliary slowing in aging is a process that can be observed in more than one strain of mouse, we also tested CBF in C57BL/6 mice. Although the baseline tracheal CBF in young C57BL/6 mice was slower than it was in BALB/c mice, we observed a similar decrease in CBF with aging. The young mice (2 mo) had a baseline CBF of 12.44 ± 0.29 Hz (n = 8); in mice aged 12 mo the baseline CBF was 10.05 ± 0.47 Hz (n = 10), and in mice aged 24 mo it was 9.46 ± 0.32 Hz (n = 9) (Fig. 1B). The differences in CBF between the mice aged 2 fig. 1. Ciliary beat frequency (CBF) diminishes with normal aging. A: tracheas from healthy BALB/c mice aged 2, 5, 12, and 24 mo were cut into rings, and CBF analysis was performed. The 12- and 24-mo-old animals had significant decreases in CBF compared with the 2- and 5-mo-old animals. B: tracheas from healthy C57BL/6 mice aged 2, 12, and 24 mo were cut into rings, and CBF analysis was performed. The young mice (2 mo) had a baseline CBF of 12.44 ± 0.29 Hz (n = 8); 12-mo baseline was 10.05 ± 0.47 Hz (n = 10); and 24-mo baseline was 9.46 ± 0.32 Hz (n = 9). The differences in CBF between the 2- and 12-mo (P < 0.01), 2- and 24-mo mice (P < 0.001), and 12- and 24-mo mice (P < 0.001) were statistically significant.
and 12 mo ($P < 0.01$), 2 and 24 mo ($P < 0.001$), and 12 and 24 mo ($P < 0.001$) were all statistically significant.

**Aged mice remain able to respond to beta agonists.** $\beta_2$ Adrenergic receptor agonists are classic agents that stimulate increases in CBF (27). To determine whether aged cilia were capable of being stimulated, tracheal rings from BALB/c mice were stimulated with the $\beta_2$-agonist procaterol (100 nM) for 5 min. At all ages, there was a significant increase in CBF with procaterol (Fig. 2). The magnitude of stimulation was similar in all age groups; with a 2.0 Hz increase in mice aged 2 mo, a 2.93-Hz increase in mice aged 12 mo, and 2.87-Hz increase in 24-mo-old animals. Despite their response to $\beta_2$ agonists, the 24-mo-old mice were stimulated only to the baseline levels of young mice.

**Aging does not diminish the number of motile cilia.** In addition to CBF, we also analyzed the number of motile cilia in each age group at baseline. We did not note any differences in the number of motile points between age groups. The tracheas of BALB/c mice aged 2 mo had 627 ± 73 motile points/high-power field (hpf) ($n = 11$); mice at 12 mo had 691 ± 153 points/hpf ($n = 8$), and mice at 24 mo had 707 ± 123 points/hpf ($n = 14$) ($P = 0.87$).

**PKCε activity is upregulated in aged mouse trachea.** To determine the mechanism of cilia slowing in aging, we measured PKCε activity in the tracheas of each age group of BALB/c mice. We found a significant increase in PKCε in mice aged 12 and 24 mo compared with mice at 2 mo (Fig. 3A). Baseline PKCε activity in mice aged 2 mo was 17.5 ± 2.2 pmol/min per mg protein ($n = 15$). In animals at 12 mo it was 43.5 ± 5.1 pmol/min per mg protein ($n = 12$) (a 2.5-fold increase), and in animals at 24 mo it was 55.9 ± 5.5 pmol/min per mg protein ($n = 8$) (a 3.2-fold increase). There was not a statistically significant difference between the 12- and 24-mo-old groups.

PKCα activity was also measured to rule out nonspecific changes in all PKC isoforms. We observed no change in PKCα activity between young and old animals ($P = 0.81$) (Fig. 3B).

**PKCε mRNA and protein is increased in aging.** PKCε activity is typically modulated allosterically; however, because aging is a long-term, complex process, we wanted to rule out increases in PKCε protein levels contributing to the increase in activity. We extracted mRNA from BALB/c tracheas and measured PKCε mRNA. We observed a clear increase in PKCε in the 24-mo-old mice ($n = 7$) compared with the mice at age 2 mo ($n = 8$; $P < 0.01$) (Fig. 4A). To determine whether this increased mRNA was transcribed into protein, we performed a PKCε ELISA on the lungs of C57BL/6 mice aged 2, 12, and 24 mo. We again observed a marked increase in PKCε protein in the mice at 12 ($n = 5$; $P < 0.01$) and 24 mo ($n = 5$; $P < 0.05$) compared with the mice at 2 mo (Fig. 4B).

**DCP-LA, a specific PKCε agonist, slows young mouse cilia.** We used the PKCε agonist 8-[2-(2-pentylcyclopropylmethyl)]-cyclpropyl]-octanoic acid (DCP-LA; Sigma) to stimulate PKCε activity in the tracheal rings of 2-mo-old animals. We have previously shown that DCP-LA is capable of specifically stimulating PKCε in bovine ciliated cells (28). We stimulated the tracheal rings of the mice with DCP-LA (10 $\mu$M) for 0–3 h, and measured CBF. We saw a significant decrease in CBF with DCP-LA exposure from 1 to 3 h (Fig. 5). After 3 h of exposure, the rings were then stimulated with procaterol. Like the aged mice, the tracheal rings that had been treated with DCP-LA were able to respond to procaterol (Fig. 5). This ensures that the DCP-LA did not simply have a nonspecific toxic effect.
DISCUSSION

Pneumonia is a very common ailment in the elderly and accounts for a significant amount of mortality and morbidity. To better prevent and treat pneumonia in the elderly, we need a better understanding of how normal aging affects the lung. It has been established that nasal CBF slows with aging (7), leading to impaired mucociliary transport (20, 24). However, the mechanisms of how normal aging influences cilia slowing and diminished mucociliary transport are unknown.

In this study, we observed age-associated cilia slowing in two different strains of mice, BALB/c and C57BL/6. Although respiratory cilia slowing has been shown in aging human nasal epithelium (7) and guinea pig trachea (10), and diminished mucociliary clearance has been shown in dogs (25), we believe we are the first to report this phenomenon in a mouse model. Like human nasal cilia, mouse tracheal cilia are slowed with aging. This gives us a new, powerful tool to investigate mechanisms of normal aging. The mouse model allows us to look at the modulation of the changes of aging in a shorter time span. It also opens up genetic approaches to the study of mucociliary clearance in aging, including the use of knockout mice.

After confirming that mice demonstrate cilia slowing in aging, we went on to show that increases in CBF could be stimulated with the addition of β-agonists (Fig. 2). This was an important finding because it shows that the ciliary changes observed in aging can be reversed. At times, the changes that occur with aging are perceived to be too complex to modulate or treat. However, in the case of cilia slowing, it may be possible to treat the changes. It would be attractive to advocate the use of β2 agonists in the treatment or prevention of pneumonia in the elderly. However, the clinical use of β2 agonists to speed cilia is limited by tachyphylaxis (11). Unlike bronchodilation, after repeated doses of β2 agonists, the cilia no longer increase their CBF. This diminished response over time makes it a poor therapeutic agent for increasing mucociliary clearance. Moreover, stimulation with a β2 agonist increased in aged animals only to baseline levels in young animals, which may not be enough. This makes it important to discern the mechanism of ciliary slowing in aging.

Although much is known about the mechanisms of CBF stimulation, very little is known about the mechanisms of slowing baseline beating. We chose to focus on PKCe because we have previously shown a slowing of CBF with stimulation of PKCe by H. influenzae (2). We demonstrated an increase in PKCe activity in aged mouse tracheas. We also measured PKCe activity, which showed no changes with aging. This shows that the changes measured in PKCe are isoform specific, and not just a general upregulation of PKC activity with aging.

To be certain that PKCe was involved in the slowing of cilia in aging, we treated young mouse tracheas with the PKCe agonist DCP-LA. We observed a similar decrease in CBF with DCP-LA stimulation as we did with aging. Like the aging tracheas, DCP-LA-triggered slowing was reversed by β2 agonist stimulation.

The increased activity of PKCe in the aging airway epithelium appears to be unique to this organ. Other researchers have shown no change in PKC isoforms in the aging brain (4), increases in PKCγ in the aging hippocampus (5), no change in PKCe in the aging myocardium (14), and no changes in PKC isoforms in lymphocytes (26). We found it very interesting to observe a sustained increase in baseline PKCe in aging. Typically, PKCe activity is modulated by phosphorylation, which is a dynamic process. The sustained nature of this upregulation led us to measure the PKCe mRNA and protein. The surprising increase in PKCe protein may at least partially explain the...
sustained upregulation of basal PKCε activity in aging. It is not known what mechanisms lead to increased transcription of PKCε; however, we speculate that oxidative stress induced by aging may play a role. For instance, oxidative stress can lead, at least transiently, to ciliary slowing through a PKC-dependent mechanism (13). It is possible that epigenetic mechanisms may be playing a role. Others have shown that changes in methylation patterns in the promoter region of PKCε can lead to changes in protein expression in the fetal heart (19, 30). It is possible that aging and oxidative stress lead to decreased methylation of the promoter region of PKCε, leading to increased transcription.

The question then becomes how increases in PKCε activity lead to the slowing in CBF. We speculate that increased PKCε activity leads to increases in phosphorylation of axonemal proteins. A good theoretical target would be an outer dynein arm protein. The outer dynein arms have been shown to play a large role in regulation of CBF (21). It is also possible that PKCε phosphorylates a ciliary membrane protein such as p37, which has been shown to be phosphorylated by PKC and lead to ciliary slowing.

Our study has several limitations. The first is that we used a mouse model. The mouse is not always representative of human disease or physiology. Clearly, future studies will need to include human tracheal tissue, because although human nasal ciliary slowing has been demonstrated in aging, human tracheal ciliary slowing has not been previously shown. In addition, although we have shown that CBF slows with aging through a PKCε-dependent mechanism, we have not demonstrated that slowing of CBF leads to diminished clearance, or increased infections.

In summary, we have demonstrated that baseline CBF slows with aging in BALB/c and C57BL/6 mice. We have shown that ciliary slowing is not just a general decline with aging, but is modulated by a specific pathway. This knowledge moves us closer to understanding why the elderly are at an increased risk of pneumonia. Future research will help identify the mechanisms of increased expression and activity of PKCε, as well as phosphorylation targets that lead to slowing. This will bring us closer to interrupting this pathway in a clinically useful way to improve mucociliary clearance in the elderly.

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DISCLOSURES
No conflicts of interest, financial or otherwise are declared by the author(s).

AUTHOR CONTRIBUTIONS

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