The Role of Short-Chain Fatty Acids, Produced by Anaerobic Bacteria, in the Cystic Fibrosis Airway

Bojana Mirković¹, Michelle A. Murray¹, Gillian M. Lavelle¹, Kevin Molloy¹, Ahmed Abdul Azim¹, Cedric Gunaratnam¹, Fiona Healy², Dubhfeasa Slattery², Paul McNally³, Joe Hatch^{4,5}, Matthew Wolfgang^{4,5}, Michael M. Tunney^{6,7}, Marianne S. Muhlebach⁸, Rosaleen Devery⁹, Catherine M. Greene¹, and Noel G. McElvaney¹

¹Respiratory Research Division, Department of Medicine, Royal College of Surgeons in Ireland, Education and Research Centre, Beaumont Hospital, Dublin, Ireland; ²Temple Street Children's University Hospital, Dublin, Ireland; ³Our Lady's Children's Hospital, Crumlin, Dublin, Ireland; ⁴Cystic Fibrosis/Pulmonary Research and Treatment Center, ⁵Department of Microbiology and Immunology, and ⁶Department of Pediatrics, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina; ⁷CF & Airways Microbiology Group and ⁸School of Pharmacy, Queen's University Belfast, Belfast, United Kingdom; and ⁹School of Biotechnology, Dublin City University, Dublin, Ireland

Abstract

Rationale: Anaerobic bacteria are present in large numbers in the airways of people with cystic fibrosis (PWCF). In the gut, anaerobes produce short-chain fatty acids (SCFAs) that modulate immune and inflammatory processes.

Objectives: To investigate the capacity of anaerobes to contribute to cystic fibrosis (CF) airway pathogenesis via SCFAs.

Methods: Samples of 109 PWCF were processed using anaerobic microbiological culture with bacteria present identified by 16S RNA sequencing. SCFA levels in anaerobic supernatants and bronchoalveolar lavage (BAL) were determined by gas chromatography. The mRNA and/or protein expression of two SCFA receptors, GPR41 and GPR43, in CF and non-CF bronchial brushings and 16HBE14o⁻ and CFBE41o⁻ cells were evaluated using reverse transcription polymerase chain reaction, Western blot analysis, laser scanning cytometry, and confocal microscopy. SCFA-induced IL-8 secretion was monitored by ELISA.

Measurements and Main Results: Fifty-seven (52.3%) of 109 PWCF were anaerobe positive. Prevalence increased with age, from 33.3% to 57.7% in PWCF younger (n = 24) and older (n = 85) than 6 years of age. All evaluated anaerobes produced millimolar concentrations of SCFAs, including acetic, propionic, and butyric acids. SCFA levels were higher in BAL samples of adults than in those of children. GPR41 levels were elevated in CFBE41o⁻ versus 16HBE14o⁻ cells; CF versus non-CF bronchial brushings; and 16HBE14o⁻ cells after treatment with cystic fibrosis transmembrane conductance regulator inhibitor CFTR(inh)-172, CF BAL, or inducers of endoplasmic reticulum stress. SCFAs induced a dose-dependent and pertussis toxin-sensitive IL-8 response in bronchial epithelial cells, with a higher production of IL-8 in CFBE41o⁻ than in 16HBE14o⁻ cells.

Conclusions: This study illustrates that SCFAs contribute to excessive production of IL-8 in CF airways colonized with anaerobes via up-regulated GPR41.

Keywords: anaerobic bacteria; cystic fibrosis; inflammation; short-chain fatty acids

(Received in original form May 14, 2015; accepted in final form August 12, 2015)

Supported by Science Foundation Ireland and the Health Research Board under grant SFI/08/US/B1676 and by the National Institutes of Health under grant 5R01 HL092964-04.

Author Contributions: Study conception: B.M., M.A.M., R.D., C.M.G., and N.G.M.; experiments and data analysis: B.M., M.A.M., G.M.L., J.H., M.W., M.M.T., C.M.G., and N.G.M.; patient recruitment: M.A.M., K.M., A.A.A., C.G., F.H., D.S., and P.M.; clinical data collection: B.M., M.A.M., G.M.L., and C.G.; interpretation of experimental results: B.M., C.M.G., and N.G.M.; drafting of the manuscript: B.M., M.S.M., C.M.G., and N.G.M.

Correspondence and requests for reprints should be addressed to Bojana Mirković, Ph.D., Department of Medicine, Royal College of Surgeons in Ireland, Education and Research Centre, Beaumont Hospital, Dublin 9, Ireland. E-mail: bojanamirkovic@rcsi.ie

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 192, Iss 11, pp 1314–1324, Dec 1, 2015

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Originally Published in Press as DOI: 10.1164/rccm.201505-0943OC on August 12, 2015

Internet address: www.atsjournals.org

At a Glance Commentary

Scientific Knowledge on the

Subject: A large number of culture-dependent and culture-independent studies have revealed a significant anaerobic bioburden in cystic fibrosis (CF) airways; however, the role of anaerobic bacteria in the pathogenesis of infection and inflammation in the CF airways remains largely underexplored.

What This Study Adds to the

Field: In the present study, we demonstrate a high prevalence of anaerobes in the airways of people with CF and show that this prevalence increases with age. Short-chain fatty acids (SCFAs) were secreted by anaerobic bacteria *in vitro* and were also detected *in vivo* in CF bronchoalveolar lavage. SCFAs induced a significant IL-8 response in bronchial epithelial cells that was more pronounced in CF than in normal bronchial epithelium. Two receptors for SCFAs, GPR41 and GPR43, were expressed in bronchial epithelial cells. However, only GPR41 was upregulated in CF compared with normal airway epithelium *in vitro* and *in vivo*. GPR41 upregulation was intrinsically driven by the lack of cystic fibrosis transmembrane conductance regulator activity and endoplasmic reticulum stress and was further corroborated by inflammatory stimuli. This study provides novel insight into the pathophysiological role of anaerobic bacteria in the CF airways.

The main cause of morbidity and mortality in cystic fibrosis (CF) is respiratory failure resulting from persistent microbial infection and neutrophil-dominated inflammation (1, 2). Bacteria such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Haemophilus influenzae*, and *Burkholderia cepacia* complex have been recognized as key pathogens in CF airway infection (3). The detection of bacterial pathogens in CF depends mostly on routine aerobic culture techniques. Consequently, the prevalence of anaerobic and other fastidious bacteria has remained largely understudied. With the implementation of enhanced

culture-dependent and culture-independent molecular diagnostics-based techniques, interest in the prevalence and potential pathogenic role of anaerobes has been sparked (4–6).

Anaerobic bacteria are present in the airways of 66–91% people with CF (PWCF) (7, 8) in numbers equal to those of *P. aeruginosa*, a well-established pathogen in the CF airways (3, 7). Studies exploring the role of anaerobes have failed to show a direct impact on airway function in CF (7, 8). However, more recent studies have associated decreasing CF airway microbial diversity, to which anaerobic bacteria contribute, with poorer lung function (9–11). Contrary to this, *Prevotella intermedia* was shown to induce a humoral immune response in PWCF and to mediate an influx of neutrophils and macrophages, thereby increasing CF airway inflammation (12). Furthermore, anaerobes belonging to the oropharyngeal flora have been shown to enhance the virulence of *P. aeruginosa* in a number of infection models (13–17).

Anaerobic bacteria comprise an integral part of the normal gut microflora, where they produce copious amounts of short-chain fatty acids (SCFAs), including acetic, propionic, and butyric acids (18). In addition to their role as fuel for intestinal epithelial cells, SCFAs modulate various processes, including cell proliferation and differentiation, hormone secretion, metabolic homeostasis, and modulation of immune and inflammatory responses (18–23), by binding to the recently deorphanized G protein-coupled receptors, GPR41 (also called free fatty acid receptor 3 or FFAR3) and GPR43 (FFAR2) and by the inhibition of histone deacetylase (HDAC) (24–26).

In the present study, we investigated the capacity of anaerobic bacteria, found in the CF airways, to induce an inflammatory response by the production of SCFAs. The results revealed that these anaerobes produce copious amounts of SCFAs that were also present in the bronchoalveolar lavage (BAL) of PWCF. SCFAs induced a dose-dependent and pertussis toxin (PTX)-sensitive IL-8 response in bronchial epithelium that was higher in CF than in normal bronchial epithelial cells. This effect coincided with increased GPR41 expression in CF compared with normal bronchial epithelial cells that was intrinsically driven by lack

of cystic fibrosis transmembrane conductance regulator (CFTR) activity and endoplasmic reticulum (ER) stress, in addition to the proinflammatory milieu of the CF airways.

Some of the results of this study were previously reported in the form of abstracts (27–29).

Methods

Comprehensive details on all methods, including quantification of SCFAs, quantification of SCFA receptors, CF and non-CF bronchial epithelial cell culture and treatments, and measurement of IL-8, are provided in the online supplement.

Study Population

A total of 109 PWCF (age range, 0–61 yr; mean age, 20.7 ± 12.1 yr; 65 males, 44 females) were recruited into this study. Seventy-three PWCF were attending Beaumont Hospital, Dublin, Ireland (age range, 18–61 yr; mean age, 27.4 ± 8.2 yr; 44 males, 29 females); 24 were attending Our Lady's Children's Hospital, Crumlin, Ireland (age range, 0–6 yr; mean age, 3.5 ± 1.6 yr; 11 males, 13 females); and 12 were attending Temple Street Children's University Hospital, Dublin, Ireland (age range, 7–17 yr; mean age, 14.6 ± 2.4 yr; 10 males, 2 females). CF was confirmed by sweat testing and/or genotyping. Only clinically stable PWCF were included in this study. Clinical stability was defined as no change in symptoms, FEV₁ within 10% of best value in the previous 6 months, and no new antibiotics started. Full informed consent was obtained from all participants or their parents before the collection of samples, and ethical approval was obtained from Beaumont Hospital, Our Lady's Children's Hospital, and Temple Street Children's University Hospital institutional review boards.

Collection and Processing of Sputum and BAL

Spontaneously expectorated sputum was collected in a sterile container that was placed into an Anaerogen Compact anaerobic pouch (Thermo Fisher Scientific, Waltham, MA) and immediately transported to an anaerobic cabinet for further processing. BAL samples were obtained from individuals undergoing diagnostic or therapeutic fiberoptic

bronchoscopy as part of routine care. In adults, 60 ml of sterile 0.9% NaCl was instilled into the right or left subsegmental bronchi. In children, flexible fiberoptic bronchoscopy was performed via a laryngeal airway mask. To prevent upper airway contamination, suction was not performed until the tip of the bronchoscope was past the carina. During the procedure, 1 ml/kg of sterile 0.9% NaCl was instilled twice in the right middle lobe and twice in the lingula, and all four samples were pooled. BAL intended for anaerobic bacterial isolation and identification was handled as described above for sputum, and BAL samples intended for SCFA determination were filtered through gauze and centrifuged at $500 \times g$ for 10 minutes at 4°C . The microbiological culture and identification of bacteria in sputum and BAL were performed as described previously (7).

Bronchial Brushing Sample Collection

All patients (control subjects and PWCF) were undergoing diagnostic or therapeutic fiberoptic bronchoscopy as part of routine care. Bronchial brushings were recovered as previously described (30).

Statistical Analysis

Data were analyzed with GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA). Unless stated otherwise, all data are presented as mean $\pm$ SEM and are representative of at least three independent experiments. Normal data were compared using Student's *t* test; nonnormal data were compared by Mann-Whitney *U* test; and, where appropriate, analysis of variance was performed. Differences were considered significant at $P < 0.05$.

Results

Anaerobic Bacteria Are Present in a High Percentage of PWCF

To determine the prevalence of anaerobic bacteria in the CF airways, 80 sputum and 29 BAL samples of 109 PWCF were collected and analyzed (one sample per patient). The presence of at least one obligate anaerobe was found in 57 (52.3%) of 109 analyzed samples. The most frequently detected obligate anaerobes belonged to the genera *Prevotella* (35.8%), *Actinomyces* (16.5%), *Veillonella* (8.3%),

and *Fusobacterium* (5.5%) (see Table E1 in the online supplement). The most commonly occurring aerobes (facultative anaerobes) belonged to the genera *Pseudomonas* (44.0%), *Staphylococcus* (41.3%), and *Streptococcus* (40.4%), the latter including *S. anginosus*, *S. constellatus*, *S. intermedius*, *S. parasanguinis*, and *S. sanguinis*. The prevalence of obligate anaerobes was greater than that of *P. aeruginosa* (43.2%). Higher prevalence of anaerobes was observed in PWCF older than 6 years of age (57.7%; $n = 85$; mean age, 25.6 ± 8.9 yr; 54 males and 31 females) than in PWCF younger than 6 years of age (33.3%; $n = 24$; mean age, 3.5 ± 1.6 yr; 11 males and 13 females) (Figure 1A), demonstrating that the anaerobic prevalence increases with age. On the basis of these data, four representative obligate anaerobes and one facultative anaerobe were selected for further work: *Prevotella melaninogenica*, *Actinomyces odontolyticus*, *Veillonella parvula*, *Fusobacterium nucleatum*, and *Streptococcus sanguinis*.

Selected Anaerobic Strains Secrete Copious Amounts of SCFAs In Vitro

To investigate whether anaerobic bacteria found in the CF airway secrete SCFAs, bacterial supernatants from five representative species grown under anaerobic conditions were collected and analyzed using gas chromatography. American Type Culture Collection and patient isolated strains of all selected species secreted a variety of SCFAs in millimolar concentrations (Figure 1B). Each individual species had its own secretion profile, which was identical for the American Type Culture Collection and patient isolate strains. For example, whereas *A. odontolyticus* and *S. sanguinis* secreted acetic acid, *V. parvula* and *F. nucleatum* displayed more complex SCFA secretomes consisting of acetic and propionic acid and acetic, propionic, and butyric acids, respectively. *P. melaninogenica*, produced the highest number of SCFAs, including acetic, *i*-butyric, 2-methylbutyric, and *i*-valeric acids, albeit in lower concentrations. Of note, *P. aeruginosa* grown under anaerobic or aerobic conditions did not secrete any SCFAs (data not shown). These data suggest that anaerobic bacteria found in the CF airway produce copious amounts of SCFAs.

SCFAs Are Present in the Airways of PWCF and Elevated in Adults Compared with Children

To investigate whether anaerobes produce SCFAs in the CF airway *in vivo*, BAL samples of adults ($n = 11$; mean age, 29.2 ± 9.3 yr; 3 males and 8 females) and children with CF ($n = 7$; mean age, 4.0 ± 2.2 yr; 2 males and 5 females) were collected and analyzed using gas chromatography. Six SCFAs detected in bacterial supernatants were also observed in the micromolar concentration range in BAL samples of adult PWCF (Figure 1C). A significant increase in acetic acid ($P < 0.001$) was observed in BAL samples of adult PWCF compared with children with CF. Similarly, propionic and *i*-butyric acids were also elevated, but the difference was not statistically significant. These results illustrate that SCFAs are present in the airways of PWCF and are elevated in adult PWCF compared with children with CF.

IL-8 Is Elevated in BAL of Adults Compared with Children with CF and Correlates with Acetic Acid

IL-8 is an inflammatory marker in the airways of PWCF that mediates neutrophil trafficking to the bronchial lumen (31). As evaluated with an IL-8 ELISA, IL-8 was markedly increased in BAL samples of adult PWCF ($n = 10$; mean age, 30.1 ± 9.7 yr; 3 males and 7 females) compared with children with CF ($n = 6$; mean age, 4.5 ± 1.9 yr; 2 males and 4 females) (Figure 1D). A correlation analysis was performed to investigate whether this phenomenon was associated with SCFA levels in CF BAL. IL-8 levels displayed a strong correlation with acetic acid in BAL samples of children and adults with CF ($n = 16$; $r^2 = 0.6409$; $P = 0.002$) (Figure 1E). Similarly, a significant correlation was also observed between IL-8 and acetic acid in BAL samples of adult PWCF ($n = 10$; $r^2 = 0.4200$; $P = 0.0427$). No significant correlation between IL-8 and other SCFAs in CF BAL was observed (data not shown). Collectively, these results showcase that elevated IL-8 levels in CF BAL are associated with high levels of acetic acid in BAL.

Anaerobic Supernatants Induce an Inflammatory Response in CF Epithelial Cells

CFBE41o[−] cells were treated with supernatants from representative species

Figure 1. Anaerobic species are found in high numbers in the cystic fibrosis (CF) airways and secrete copious amounts of short-chain fatty acids (SCFAs) *in vitro* and *in vivo*. (A) The percentage of anaerobe-positive cultures in children with CF up to 6 years of age ($n = 24$; mean age, 3.5 ± 1.6 yr; 11 males and 13 females) and people with cystic fibrosis (PWCF) older than 7 years of age ($n = 85$; mean age, 25.6 ± 8.9 yr; 54 males and 31 females). Sputum or bronchoalveolar lavage (BAL) samples of 109 PWCF were collected and screened for the presence of anaerobic bacteria as stated in the METHODS section. For the sake of clarity, only four genera of the most frequent obligate anaerobes (*Prevotella*, *Actinomyces*, *Veillonella*, and *Fusobacterium*) and the most commonly occurring aerobes (facultative anaerobes) belonging to the genera *Pseudomonas*, *Staphylococcus*, and *Streptococcus* are shown. (B) SCFA levels in bacterial supernatants from American Type Culture Collection (ATCC) and patient isolate strains of *Prevotella melaninogenica*, *Actinomyces odontolyticus*, *Veillonella parvula*, *Fusobacterium nucleatum*, and *Streptococcus sanguinis* as determined by gas chromatography. Data are presented as mean $\pm$ SEM ($n \geq 3$). (C) SCFA levels in BAL samples of children with CF ($n = 7$; mean age, 4.0 ± 2.2 yr; 2 males and 5 females) and adults with CF ($n = 11$; mean age, 29.2 ± 9.3 yr; 3 males and 8 females). (D) IL-8 levels in BAL samples of children with CF ($n = 6$; mean age, 4.5 ± 1.9 yr; 2 males and 4 females) and adults with CF ($n = 10$; mean age, 30.1 ± 9.7 yr; 3 males and 7 females) as evaluated with an IL-8 ELISA. (E) Correlation between IL-8 and acetic acid in BAL samples of PWCF ($n = 16$; $r^2 = 0.6409$; $P = 0.002$). $**P \leq 0.01$; $***P \leq 0.001$.

and monitored for IL-8 secretion using ELISA. All five strains induced a dose- and PTX-dependent IL-8 response in CFBE410⁺ cells (Figure 2 and Figures E1 and E2). The highest IL-8 response was observed with *V. parvula* and *F. nucleatum* supernatants, which were shown to produce high amounts of acetic, propionic, and butyric acids. Interestingly, the IL-8 response induced by *V. parvula* and *F. nucleatum* was equal to or greater than that induced by the major CF pathogens *S. aureus* and *P. aeruginosa*. When CFBE410⁺ cells were treated with *S. sanguinis* or *A. odontolyticus*, which secreted acetic acid only, a less pronounced IL-8 response was observed and higher concentrations of bacterial supernatant (5% and 10%) were needed to achieve significant cytokine production (Figure E1). *P. melaninogenica* displayed an intermediate but significant impact on IL-8 production. Upon stimulation of 16HBE14o⁺ cells with anaerobic supernatants, only *F. nucleatum* induced a significant IL-8 response compared with baseline levels (Figure 2). Furthermore, anaerobe-induced IL-8 responses in 16HBE14o⁺ cells were strikingly lower than in CFBE410⁺ cells, suggesting that the CF epithelium is more susceptible to stimulation by anaerobic supernatants.

SCFAs Generate a Notable IL-8 Response in CF Epithelial Cells

To elucidate whether SCFAs in anaerobic supernatants contribute to IL-8 secretion, bronchial epithelial cells were treated with SCFAs and analyzed for IL-8 secretion. The influence of SCFAs on cell viability was evaluated to exclude any SCFA-induced cytotoxicity (Figure E3), and in the following experiments the cells were treated with nontoxic concentrations of SCFAs. All SCFAs, apart from 2-methylbutyric acid, induced a significant and a dose-dependent IL-8 response from CFBE410⁺ cells (Figure 3) with the following potency in descending order: butyric acid (half-maximal effective concentration [EC_{50}] = 0.170 mM; $R^2 = 0.8816$), *i*-valeric acid ($EC_{50} = 1.0$ mM; $R^2 = 0.7711$), propionic acid ($EC_{50} = 4.6$ mM; $R^2 = 0.9107$), acetic acid ($EC_{50} = 15.5$ mM; $R^2 = 0.8443$), *i*-butyric acid ($EC_{50} = 64$ mM; $R^2 = 0.8304$). At the same time, no basal or SCFA-stimulated IL-1 β levels were detected in CFBE410⁺ supernatants (data

Figure 2. Supernatants from anaerobic bacteria promote a proinflammatory response in cystic fibrosis airway epithelial cells. 16HBE14o⁻ and CFBE41o⁻ cells were treated with 5% bacterial supernatants or growth medium as a control for 24 hours, and IL-8 secretion was assessed with an ELISA. All data are presented as mean $\pm$ SEM of at least three independent experiments. ** $P \leq 0.01$; *** $P \leq 0.001$. The dotted line represents the normalized IL-8 production in the presence of control medium.

not shown). Additionally, a small but insignificant increase in SCFA-induced IL-6 over baseline was observed in CFBE41o⁻ supernatants (Figure E4). However, this response was markedly lower than with IL-8, confirming that IL-8 is the predominant proinflammatory cytokine induced by SCFAs in CF bronchial epithelial cells. When CFBE41o⁻ cells were treated with a combination of SCFAs mimicking the complex SCFA secretomes of *P. melaninogenica*, *F. nucleatum*, and *V. parvula*, no synergistic effect on IL-8 secretion was observed (Figure E5). SCFAs also induced a dose-dependent IL-8 response in 16HBE14o⁻ cells (Figure 3); however, IL-8 secretion was significantly less pronounced in 16HBE14o⁻ cells than in CFBE41o⁻ cells, corroborating our earlier observations with anaerobic supernatants. These results confirm that SCFAs in anaerobic supernatants are at least in part responsible for the

inflammatory response in bronchial epithelial cells *in vitro*.

Of note, upon incubation of representative SCFAs (acetic, propionic, and butyric acids) with human serum albumin or α_1 -antitrypsin, no changes in intrinsic fluorescence of either proteins were observed (Figure E6), suggesting that SCFAs do not bind human serum albumin or α_1 -antitrypsin. Moreover, the latter also failed to attenuate the SCFA-induced IL-8 response in CFBE41o⁻ cells (Figure E7).

SCFA-mediated Release of IL-8 by CFBE41o⁻ Cells Is GPR41 Dependent

Previous studies have shown that GPR41 couples exclusively through the PTX-sensitive G $_{\alpha i/o}$ family, whereas GPR43 displays dual coupling through G $_{\alpha i/o}$ and PTX-insensitive G $_{\alpha q}$ protein families (24, 25). To explore whether SCFA-mediated IL-8 release was indeed mediated by SCFA

receptors, CFBE41o⁻ cells were treated with PTX before SCFAs. PTX (250 ng/ml) significantly attenuated IL-8 secretion in CFBE41o⁻ cells induced by all evaluated SCFAs (Figure 4A). Higher PTX concentrations (500 and 1,000 ng/ml) did not fully restore the IL-8 baseline levels (Figure E8). These data suggest the involvement of PTX-sensitive receptors GPR41 and GPR43 in SCFA-mediated cytokine response in CFBE41o⁻ cells.

To further confirm the involvement of GPR41 in SCFA-mediated IL-8 response, a small interfering RNA (siRNA)-knockdown approach was employed. siRNA (80 nM)-mediated knockdown of GPR41 was confirmed using reverse transcription polymerase chain reaction and Western blot analysis 48 hours after transfection (Figure 4B and 4C). The reduction of GPR41 protein expression ($\sim 50\%$), although not complete, was sufficient to produce a significant decrease in the IL-8 production induced by acetic acid (10 mM), propionic acid (1 mM), and butyric acid (1 mM) (Figure 4D). Collectively, these data corroborate the involvement of GPR41 in SCFA-mediated IL-8 release by CF bronchial epithelial cells.

GPR41 Expression Is Increased in CF Compared with Normal Bronchial Epithelial Cells

To confirm the expression of SCFA receptors in human bronchial epithelium, mRNA and protein levels of GPR41 and GPR43 were evaluated. Western blot analysis revealed that the GPR41 receptor was expressed by both 16HBE14o⁻ and CFBE41o⁻ cells, with significantly higher protein expression in CFBE41o⁻ cells than in 16HBE14o⁻ cells ($P < 0.001$) (Figure 5A). GPR43 was expressed in 16HBE14o⁻ and CFBE41o⁻ cells at equivalent levels (Figure 5A). The cell surface expression of SCFA receptors was further examined with laser scanning cytometry and confocal microscopy. Staining for the GPR41 receptor was significantly increased on the surface of CFBE41o⁻ cells compared with 16HBE14o⁻ cells as assessed with laser scanning cytometry ($55.5 \pm 19.5\%$; $P < 0.001$) (Figure 5B). Although both cell lines displayed intracellular staining for GPR41 receptor as visualized with confocal microscopy (Figure 5C), a significant increase in colocalization of

Figure 3. Short-chain fatty acids (SCFAs) induce a dose-dependent IL-8 response in bronchial epithelial cells. 16HBE14o⁻ and CFBE41o⁻ cells were treated with increasing concentrations of SCFAs for 24 hours and monitored for IL-8 production using an ELISA. All data are presented as means $\pm$ SEM of at least three independent experiments. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. The dotted line represents the normalized IL-8 production in the presence of control medium.

the GPR41 receptor with the plasma membrane marker Na⁺/K⁺-ATPase was observed in CFBE41o⁻ cells ($11.9 \pm 2.0\%$) compared with 16HBE14o⁻ cells ($6.9 \pm 1.1\%$, $P = 0.0434$). No differences in GPR43 cell surface expression were detected in 16HBE14o⁻ and CFBE41o⁻ cells when analyzed with laser scanning cytometry or confocal microscopy (Figure E9). These results suggest that although GPR41 and GPR43 are both present in bronchial epithelial cells, only GPR41 is up-regulated at the surface of CF epithelial cells compared with normal bronchial epithelial cells.

To investigate whether *GPR41* mRNA was also increased in CFBE41o⁻ cells,

quantitative reverse transcription polymerase chain reaction was performed. *GPR41* mRNA levels were significantly increased in CFBE41o⁻ compared with 16HBE14o⁻ cells ($P < 0.001$) (Figure 5D). The *in vitro* trend was also observed *in vivo* as an increase in *GPR41* mRNA levels in bronchial brushings from PWCF compared with non-CF controls ($P = 0.0064$) (Figure 5E).

Endoplasmic Reticulum Stress Up-regulates GPR41 Expression in CF Bronchial Epithelial Cells *In Vitro*

Previous studies have associated dysfunctional CFTR with ER stress and activation of the unfolded protein response (UPR) (32). To elucidate whether the latter

could have an impact on GPR41 expression, 16HBE14o⁻ cells were treated with tunicamycin (2 μ g/ml) or thapsigargin (2 μ M), which are well-established inducers of ER stress and UPR (33). ER stress and UPR induction by these compounds was confirmed by monitoring the expression of glucose-regulated proteins 78 and 94 (Figure E10). Tunicamycin and thapsigargin significantly increased GPR41 protein levels in 16HBE14o⁻ cells ($P = 0.0297$ and $P = 0.0198$, respectively) (Figure 6A). These data suggest that ER stress and UPR may have a role in GPR41 up-regulation in CF bronchial epithelial cells.

Dysfunctional CFTR and the Inflammatory CF Environment Promote GPR41 Up-regulation in CF Bronchial Epithelial Cells *In Vitro*

To explore the possibility that GPR41 up-regulation in the CF airway epithelium could also be associated with reduced CFTR activity, 16HBE14o⁻ cells were treated with a CFTR inhibitor, CFTR (inh)-172 (10 μ M), and subsequently evaluated for *GPR41* mRNA expression. CFTR inhibition significantly increased *GPR41* mRNA levels in 16HBE14o⁻ cells ($P = 0.0166$) (Figure 6B), matching them to levels found in CFBE41o⁻ cells (Figure 5). Similarly, 10% CF BAL caused a significant up-regulation of *GPR41* mRNA in 16HBE14o⁻ cells ($P < 0.001$) (Figure 6B). These findings were translated to the protein level, as both CFTR(inh)-172 and 10% CF BAL significantly elevated GPR41 protein levels in 16HBE14o⁻ cells ($P = 0.0233$ and $P = 0.0098$, respectively) (Figure 6C). Similarly, GlyH-101, another inhibitor of CFTR, also increased GPR41 protein levels (data not shown). These results imply that reduced CFTR activity may be associated with GPR41 up-regulation in CF bronchial epithelial cells *in vitro*, an effect that is also driven by the inflammatory CF airway milieu.

Discussion

Recent advances in culture-independent molecular techniques for bacterial identification have sparked a renewed interest in the role of anaerobic bacteria in the CF airways. Therefore, we aimed to explore the contribution of anaerobes via SCFAs to the pathogenesis of CF airway

Figure 4. Pertussis toxin and GPR41 small interfering RNA (siRNA) attenuate short-chain fatty acid (SCFA)-mediated IL-8 secretion in cystic fibrosis bronchial epithelial cells. (A) CFBE41o⁻ cells were treated with pertussis toxin (PTX; 250 ng/ml) and increasing concentrations of SCFAs for 24 hours. Afterward, cell supernatants were monitored for IL-8 secretion using an ELISA. CFBE41o⁻ cells were transfected with GPR41 or control siRNA and were evaluated for GPR41 mRNA and protein levels 48 hours after transfection using quantitative reverse transcription polymerase chain reaction and Western blot analysis, respectively. (B) mRNA expression of GPR41 relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was determined using the 2^{-ΔΔCt} cycle threshold method, and the results are presented as fold differences. (C) Whole cell lysates were separated by 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to a polyvinylidene fluoride membrane, and detected with anti-GPR41 antibody. Signal intensities of all bands were quantified by densitometry and normalized against GAPDH, which served as a loading control. Bar graph depicts densitometric analysis of GPR41 expression. All data are presented as means ± SEM (n ≥ 3). (D) CFBE41o⁻ cells were transfected with GPR41 or control siRNA (80 nM) 24 hours before treatment with acetic acid (10 mM), propionic acid (1 mM), and butyric acid (1 mM) for an additional 24 hours. Cell supernatants were monitored for IL-8 secretion using an ELISA. The results shown are representative of three independent experiments. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

disease. In the present study, we show that anaerobic bacteria commonly found in the CF airways secrete SCFAs *in vitro* and *in vivo* and, by acting through SCFA receptors, mediate IL-8 release, promoting an inflammatory response in the CF airway.

Similarly to previous studies (7, 8, 16), our cohort of PWCF displayed a high prevalence of anaerobic bacteria (52.3%). This number was greater than that of *P. aeruginosa* (43.2%), an established CF airway pathogen, confirming that anaerobes are prevalent in the CF airways and may play a role during the course of the disease. Indeed, the percentage of PWCF positive for anaerobes increased with their age. Five representative species that were selected on the basis of their prevalence, secreted high quantities of SCFAs, with each displaying a distinct SCFA secretome (Figure 1B). SCFAs were also detected in CF BAL and were elevated in BAL samples of adult PWCF compared with children with CF (Figure 1C). This suggests that, alongside anaerobes, SCFAs also increase with age. Although SCFAs were previously studied in the context of allergic airway inflammation (34), the present study is the first, to our knowledge, to prove the presence of SCFAs in the lower airways.

CF airway disease is characterized by an exaggerated neutrophil influx that plays a key role in tissue damage and disease progression (35). We have previously shown that IL-8, a potent neutrophil chemotactic agent, is readily secreted from CF airway epithelium by various stimuli (36–38) and is found in high concentrations in CF BAL (31). Anaerobic supernatants induced a potent and dose-dependent IL-8 response that was more pronounced in CF than in normal bronchial epithelium (Figure 2 and Figure E1). Similarly, SCFAs generated a dose-dependent IL-8 response in bronchial epithelial cells that was higher in CF than in normal bronchial epithelial cells (Figure 3), suggesting that SCFAs in anaerobic supernatants contribute to the IL-8 response and may have a detrimental role in the CF airways by promoting neutrophil mobilization.

In addition to the activation of airway epithelial cells to produce cytokines, SCFAs are known to display a plethora of other effects. SCFAs were recently shown to act as chemotactic agents for neutrophils via GPR43 (39). Butyric acid was also

Figure 5. Short-chain fatty acid receptor GPR41 is upregulated at the surface of cystic fibrosis (CF) bronchial epithelial cells. (A) A representative Western blot depicting GPR41 and GPR43 protein expression in 16HBE14o⁻ and CFBE41o⁻ cells. Whole cell lysates were separated by 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to a polyvinylidene fluoride membrane, and detected with anti-GPR41 or anti-GPR43 antibody. Signal intensities of all bands were quantified by densitometry and normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which served as a loading control. Bar graph depicts densitometric analysis of GPR41 expression in 16HBE14o⁻ and CFBE41o⁻ cells. Data are shown as means $\pm$ SEM ($n \geq 3$). (B) GPR41 cell surface staining was analyzed in CFBE41o⁻ and 16HBE14o⁻ cells using laser scanning cytometry as stated in the METHODS section. Histograms representative of three independent experiments are shown. *Inset values* represent fold increases in mean fluorescence intensity between cells stained with primary anti-GPR41 and secondary antibody (*black line*) and control cells stained only with the secondary antibody (*gray line*). (C) 16HBE14o⁻ or CFBE41o⁻ cells were grown on coverslips and probed with anti-GPR41 and anti-Na⁺/K⁺-ATPase antibody, respectively, followed by fluorescein isothiocyanate (FITC)-labeled and Alexa Fluor 568 secondary antibody, respectively. Controls were labeled with secondary antibodies only and are shown in Figure E11. Cell nuclei were visualized using 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; *blue*), GPR41 and Na⁺/K⁺-ATPase are represented as *green* and *red* fluorescence, and colocalization is shown in *white*. Images shown are representative of three independent experiments. Scale bar, 5 μ m. Quantitative reverse transcription polymerase chain reaction was performed (D) *in vitro* using CFBE41o⁻ and 16HBE14o⁻ epithelial cells and (E) *in vivo* using bronchial brushings from people with cystic fibrosis ($n = 3$; mean age, 23.0 ± 4.0 yr; 2 males and 1 female) and control subjects without CF ($n = 3$; mean age, 56.3 ± 9.5 yr; 3 females). Expression of GPR41 relative to GAPDH was determined using the $2^{-\Delta\Delta C_t}$ cycle threshold method, and the results are presented as fold differences. Data are presented as means $\pm$ SEM ($n \geq 3$). ** $P \leq 0.01$; *** $P \leq 0.001$.

reported to impair neutrophil reactive oxygen species production, phagocytosis, and microbial killing (40). This suggests that, in addition to IL-8-mediated recruitment of neutrophils, SCFAs may display a direct effect on neutrophil migration to the CF airways and on their function.

Both SCFA receptors, GPR41 and GPR43, couple to the PTX-sensitive $G_{\alpha i/o}$

family of proteins, but only GPR43 also couples to the PTX-insensitive $G_{\alpha q}$ family of proteins (24, 25). PTX blocks the signaling mediated by $G_{\alpha i/o}$ proteins, and PTX treatment significantly reduced the SCFA-mediated IL-8 response (Figure 4), confirming that SCFAs mediate their effects via SCFA receptors GPR41 and GPR43. However, increasing the concentration of PTX did not return the

levels of IL-8 production to baseline after stimulation by propionic and butyric acids (Figure E8). Propionic and butyric acids are well-established inhibitors of HDAC (41), and previous studies have shown that butyric acid induces an IL-8 response in human intestinal epithelial cells by inhibiting HDAC activity and promoting histone acetylation (26). Therefore, SCFA-induced IL-8 response in bronchial

Figure 6. The mechanism of GPR41 upregulation in cystic fibrosis (CF) bronchial epithelial cells. (A) 16HBE14o⁻ cells were treated with thapsigargin (2 μ M) or tunicamycin (2 μ g/ml) for 24 hours, and GPR41 protein levels were evaluated by Western blot analysis. Whole cell lysates were separated by 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to a polyvinylidene fluoride membrane, and detected with anti-GPR41 antibody. Signal intensities of all bands were quantified by densitometry and normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which served as a loading control. Bar graph depicts densitometric analysis of the GPR41 expression. (B) 16HBE14o⁻ cells were treated with a cystic fibrosis transmembrane conductance regulator inhibitor (CFTR[inh]-172; CFTRi) (10 μ M) or 10% CF bronchoalveolar lavage (BAL) for 24 hours and 6 hours, respectively, and the *GPR41* mRNA levels were evaluated using quantitative reverse transcription polymerase chain reaction as described in the METHODS section. Expression of *GPR41* relative to *GAPDH* was determined using the $2^{-\Delta\Delta C_t}$ cycle threshold method, and the results are presented as fold differences. (C) 16HBE14o⁻ cells were treated with CFTR[inh]-172 (20 μ M) for 48 hours or 10% CF BAL for 24 hours, and GPR41 protein levels were evaluated with Western blot analysis as described above. Data are presented as means $\pm$ SEM of at least three independent experiments. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. DMSO = dimethyl sulfoxide; Thaps. = thapsigargin; Tun. = tunicamycin.

epithelial cells may also be mediated by HDAC inhibition in addition to SCFA receptors. Nevertheless, even a partial reduction (~50%) of GPR41 protein expression induced by siRNA silencing was sufficient for significant inhibition of SCFA-mediated IL-8 production (Figure 4D), corroborating the role of GPR41 in SCFA-mediated IL-8 release by CF bronchial epithelial cells.

GPR41 expression is increased in CF bronchial epithelium *in vitro* and *in vivo* (Figure 5), and the mechanism of up-regulation was subsequently investigated. F508del-CFTR is the most common cause of CF. It causes misfolding

of CFTR and its subsequent degradation after synthesis, leading to a lack of the functional CFTR on the cell surface (42). Treatment with CFTR[inh]-172, a CFTR inhibitor, increased mRNA and protein levels of GPR41 in 16HBE14o⁻ cells (Figure 6B and 6C), suggesting that the lack of CFTR activity could be associated with GPR41 up-regulation in CF bronchial epithelial cells and that the mechanism could be related to defective chloride ion and/or bicarbonate conductance. Previously, we demonstrated altered expression of microRNAs (miRNAs) in CF (30). miR-182, miR-23b, and miR-544 were shown to be decreased in CF bronchial

brushings (30), and we identified them as targeting *GPR41* mRNA by using various miRNA target prediction databases (43, 44). However, overexpression of these miRNAs failed to reduce *GPR41* mRNA and protein expression in CFBE41o⁻ cells (data not shown), excluding the possibility of regulation of GPR41 expression by these miRNAs. Accumulation of misfolded F508del-CFTR in the ER causes ER stress that activates UPR, a signal transduction pathway to alleviate this stress and restore ER homeostasis (32, 45, 46). Exposure of 16HBE14o⁻ cells to tunicamycin or thapsigargin, well-known inducers of ER stress and UPR, increased protein expression of GPR41 (Figure 6A), implying that the ER stress and UPR may also, independent of CFTR activity, contribute to GPR41 up-regulation. The CF airway also harbors high concentrations of proinflammatory markers, including proteases and chemokines (31). Treatment of 16HBE14o⁻ cells with CF BAL increased GPR41 mRNA and protein levels (Figure 6B and 6C), suggesting that, in addition to intrinsic up-regulation of GPR41 in CF, the latter could further be corroborated by the inflammatory milieu of the CF airways.

In conclusion, we propose a novel mechanism by which anaerobic bacteria, through the production of SCFAs, contribute to the inflammatory environment in the CF airways, in this way potentially amplifying mobilization of neutrophils, tissue destruction, and disease progression. These data suggest that eradication of anaerobes in PWCF may be beneficial by resolving the excessive inflammation brought on by these microorganisms. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: The authors thank David Cunningham for technical assistance with gas chromatography and the patients with cystic fibrosis for taking part in this study.

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