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Hyperglycaemia in cystic fibrosis adversely affects BK channel function critical for mucus clearance

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ABSTRACT Large-conductance, Ca^{2+}-activated, voltage-dependent K^+ (BK) channel function is critical for adequate airway hydration and mucociliary function. In airway epithelia, BK function is regulated by its γ-subunit, leucine-rich repeat-containing protein 26 (LRRC26). Since patients with cystic fibrosis (CF)-related diabetes mellitus (CFRD) have worse lung function outcomes, this study determined the effects of hyperglycaemia on BK function in CF bronchial epithelial (CFBE) cells *in vitro* and evaluated the correlation between glycaemic excursions and mRNA expression of *LRRC26* in the upper airways of CF and CFRD patients.

CFBE cells were redifferentiated at the air–liquid interface (ALI) in media containing either 5.5 mM or 12.5 mM glucose. BK activity was measured in an Ussing chamber. Airway surface liquid (ASL) volume was estimated by meniscus scanning and inflammatory marker expression was measured by quantitative real-time PCR and enzyme-linked immunosorbent assay (ELISA). CF patients were assessed by 7 days of continuous glucose monitoring (CGM). *LRRC26* mRNA expression was measured by quantitative real-time PCR from nasal cells obtained at the end of glucose monitoring.

BK currents were significantly decreased in CFBE cells cultured under high glucose. These cells revealed significantly lower ASL volumes and increased inflammation, including the receptor for advanced glycation endproducts (RAGE), compared to cells cultured in normal glucose. *In vivo*, nasal cell expression of *LRRC26* mRNA was inversely correlated with hyperglycaemic excursions, consistent with the *in vitro* results.

Our findings demonstrate that hyperglycaemia induces inflammation and impairs BK channel function in CFBE cells *in vitro*. These data suggest that declining lung function in CFRD patients may be related to BK channel dysfunction.

Introduction

Cystic fibrosis (CF)-related diabetes mellitus (CFRD) is a major predictor of worse lung function and affects ~20% of adolescents and >40% of adults with CF [1–3]. However, little is known about the mechanisms by which elevated glucose levels lead to worse lung function.

Advances in care and therapeutic treatments for CF patients have led to a dramatic increase in life expectancy. However, the consequence of prolonged life is an increase in the prevalence of age-related and CF-related comorbidities that can worsen pulmonary function. In fact, CFRD is the most common CF comorbidity and is associated with worse lung function, increased number and severity of pulmonary exacerbations, increased risk of infection by *Pseudomonas aeruginosa* and poorer prognosis compared to CF patients without diabetes [4]. Thus, it is imperative to initiate mechanistic studies that could be translated into lung function preservation trials in CFRD beyond glycaemic control.

Hyperglycaemia initiates pro-inflammatory signalling pathways [5], including the formation of advanced glycation endproducts (AGE). These bind to the receptor for advanced glycation endproducts (RAGE or AGER), a member of the immunoglobulin superfamily of cell-surface receptors, which functions as a pattern-recognition receptor and engages pro-inflammatory signalling pathways when activated [6]. RAGE is highly expressed in the lung and is also important for cell adhesion and epithelial repair [7, 8]. RAGE signalling has been implicated in the pathogenesis of a number of respiratory disorders, including chronic obstructive pulmonary disease (COPD), asthma and pulmonary fibrosis [9]. In CF patients, even more so in CFRD patients, levels of RAGE ligands in the lung inversely correlate with lung function [10, 11].

It is known that inflammation and hyperglycaemia impair airway epithelial ion channel function [12] and thereby possibly contribute to respiratory decline in CFRD patients, but little is known about mechanism. Here, we specifically studied the impact of hyperglycaemia on apically expressed large-conductance, Ca^{2+}-activated, voltage-dependent K^+ (BK) channels because they are critical to maintaining mucus clearance in CF bronchial epithelial (CFBE) cells [13].

Our data reveal that airway surface liquid (ASL) volume reduction under hyperglycaemic conditions, in CFBE cells *in vitro*, correlated with reduced BK channel function and increased expression of RAGE, which could initiate pro-inflammatory signalling cascades under conditions of persistent hyperglycaemia [14]. BK channel dysfunction was likely due to a decrease in expression of leucine-rich repeat-containing protein 26 (LRRC26), the BK γ-subunit needed for its function in non-excitable cells [15, 16]. Furthermore, continuous monitoring of glucose levels in CF patients with or without CFRD over a 1-week period revealed that hyperglycaemic excursions inversely correlated with mRNA expression of *LRRC26*.

Methods

Lungs

All non-CF human bronchial epithelial (NHBE) cells were from nonsmoking donors with no known pre-existing airway diseases or lung diseases, whose lungs were not used for transplant by the Life Alliance Organ Recovery Agency (University of Miami, Miami, FL, USA), the LifeCenter Northwest (Seattle, WA, USA), or the Midwest Transplant Network (Kansas City, KS, USA). Organ donation was performed on donors who had consented appropriately with the organ procurement agencies. Thus, cell and tissue use are not considered human subjects research (deceased individuals). Non-CF lung donor information is presented in supplementary table S1. Most CFBE cells stemmed from explanted lungs from patients undergoing lung transplantation, who gave with appropriate consent and which was approved by the University of Miami's Institutional Review Board (IRB). Lungs from one deceased CF donor were procured through LifeCenter Northwest. CF lung donor information is presented in supplementary table S2.

Cell culture

Culturing of NHBE and CFBE cells at the air–liquid interface (ALI) was performed as described (supplementary material) [17–19]. Cells were re-differentiated at an ALI in media containing 5.5 mM or 12.5 mM glucose for a minimum of 3 weeks before experiments were performed. Glucose levels in media were measured with a calibrated OneTouch Verio meter (LifeScan, Malvern, PA, USA). For high mobility group box 1 (HMGB1) experiments, 100 ng·mL^{-1} recombinant human HMGB1 (H4652, MilliporeSigma, Burlington, MA, USA) was added to the basolateral media 24 h before Ussing chamber and ASL volume measurements.

Quantitative PCR

NHBE and CFBE cells were lysed and total RNA was isolated using the E.Z.N.A. Total RNA Kit (Omega Bio-tek, Norcross, GA, USA). Quantitative real-time PCR was performed as described [20, 21] using TaqMan Gene Expression Assays (ThermoFisher Scientific, Waltham, MA, USA) for *AGER* (Hs00153957_m1), *IL-1*β (Hs01555410_m1), *IL-6* (Hs00985639_m1), *IL-8* (Hs00174103), *KCNMA1*

(Hs00266938_m1), *KCNN4* (Hs00158470_m1), *LRRC26* (Hs02385555_g1), *MMP-9* (Hs00234579_m1), *COX-2* (Hs00153133_m1) and *TGF-β1* (Hs00998133_m1), and normalised to reference gene *GAPDH* (GAPDH: glyceraldehyde 3-phosphate dehydrogenase).

Enzyme-linked immunosorbent assay

Basolateral media from CFBE cells cultured in ALI media with starting glucose concentrations of 5.5 mM or 12.5 mM were collected 48 h after the previous media change. Media samples were analysed using the Ella Automated Immunoassay System and Simple Plex cartridge-based immunoassay for interleukin (IL)-1β, IL-6 and Il-8 (Bio-Techne Corp., Minneapolis, MN, USA).

Ussing chamber

CF transmembrane conductance regulator (CFTR) and large conductance, Ca^{2+}-activated and voltage-dependent K^+ channel (BK) activities were recorded in Ussing chambers as previously described (supplementary material) [16, 22]. Ussing chamber experiments were performed 24 h after apical wash and basolateral media change unless otherwise noted. CFTR and BK measurements in NHBE cells on Snapwell inserts (Corning Inc, Corning, NY, USA) were conducted using 1.12 cm^2 aperture sliders (P2302, Physiologic Instruments, San Diego, CA, USA). BK measurements in CFBE cells on Transwell inserts (Corning Inc) were conducted using 0.10 cm^2 aperture sliders (P2303, Physiologic Instruments). The smaller aperture size likely allows for greater epithelial uniformity, which may account for the larger BK currents observed in CFBE cells.

Airway surface liquid volume

ASL volume estimation was performed by meniscus scanning as previously published [21, 23]. Briefly, the apical surface of the cells was washed with Dulbecco's phosphate-buffered saline (Corning Inc) and ASL volumes were measured after 24 h. Basolateral media was replenished with ALI media containing either 5.5 mM or 12.5 mM glucose and ASL volumes were measured again after another 24 h. Change in ASL (ΔASL) represents the difference between these two measurements.

Human Study approval

The study was approved by the University of Kansas Medical Center IRB and informed consent was obtained from each participant.

Human subjects

Patients aged 18 years or older in the University of Kansas Health System, with known CF (with or without CFRD), were enroled in an observational cohort study (see supplementary material for additional enrolment criteria). CFRD was defined as fasting blood glucose >126 mg·dL^{-1} or 2 h blood glucose >200 mg·dL^{-1} during an oral glucose tolerance test (OGTT). Those with a normal OGTT (<126 mg·dL^{-1} at baseline or <140 mg·dL^{-1} at 2 h) or an impaired OGTT (<126 mg·dL^{-1} at baseline or 140–200 mg·dL^{-1} at 2 h) were defined as CF only. Demographics and baseline clinical variables were collected at the initial study visit and haemoglobin A1C was measured from capillary blood using A1CNow+ (PTS Diagnostics, Whitestown, IN, USA). Study participants wore blinded continuous glucose monitors (Dexcom G6, Dexcom Inc., San Diego, CA, USA) for 5–10 days. Nasal cells were collected from study participants at the time of monitor removal using sterile cytology brushes (Medical Packaging Corp., Camarillo, CA, USA) as described in the supplementary material.

Continuous glucose monitoring analyses

Continuous glucose monitoring (CGM) variables were calculated using the R statistical package "cgmanalysis" (The R Project for Statistical Computing, www.r-project.org) [24]. Imputation was used to fill missing intervals <20 min in length and, for those intervals >20 min in length, the corresponding 24 h period was censored from the final analysis. CGM variables of interest were selected due to being previously associated with outcomes in CFRD (mean amplitude of glycaemic excursion (MAGE) and percentage time glucose >140 mg·dL^{-1}) or due to being reflective of hyperglycaemic excursions (glucose management indicator (GMI), percentage time glucose >200 mg·dL^{-1} and area under the curve (AUC) of glucose >180 mg·dL^{-1}) [25, 26].

Statistical analyses

For *in vitro* studies, data are shown as mean±standard error of the mean (SEM). Data that followed normal distribution by Shapiro–Wilk normality testing were analysed by parametric tests. Otherwise, nonparametric statistical analyses were performed. Multiple groups were analysed by one-way ANOVA followed by the appropriate *post hoc* test. Two groups were compared with a paired or unpaired t-test as appropriate. Results were considered statistically significant at p<0.05. Clinical study data are shown as dot

plot/bar graph combinations with mean±SEM, or as trend lines with 95% confidence intervals (CIs). Differences between two groups were compared by parametric or nonparametric tests as indicated in the figure captions depending on whether the data passed Shapiro–Wilk testing. Pearson's correlation coefficient was used to assess correlation between continuous variables. Results were considered statistically significant at p<0.05. All analyses were performed using Prism 8 (GraphPad Software, San Diego, CA, USA).

Results

Hyperglycaemia differentially affects ion channel function in NHBE cells in vitro

NHBE cells cultured at the ALI were exposed to high levels of glucose in the basolateral media (12.5 mM or 225 mg·dL^{-1}). Glucose levels decreased over time, reaching normal blood levels 48 h after media change (figure 1a). BK channel conductance was measured at 24 h and 72 h after media change, corresponding to time points with high glucose conditions (24 h range: 7.2–12.5 mM) and normal glucose conditions (72 h range: 3.1–5.7 mM), respectively. BK channel currents were reduced by 50% in NHBE cells under high glucose conditions (at 24 h) compared to normal glucose conditions (at 72 h) (figures 1b and 1c).

BK channels comprise the pore-forming α-subunit, encoded by the *KCNMA1* gene and the auxiliary β- and γ-subunits [28]. BK channel function in airway epithelial cells requires the γ-regulatory subunit LRRC26 and is directly related to *LRRC26* expression [16]. We found that NHBE cells under high glucose conditions expressed lower levels of *LRRC26* mRNA compared to cells under normal glucose conditions (figure 1d), consistent with the functional BK channel data. On the other hand, *KCNMA1* mRNA expression levels remained unchanged (figure 1e). NHBE cells under high glucose conditions showed a significantly greater response to mallotoxin, a BK channel opener that is more effective in the absence of LRRC26 (figures 1f and 1g), providing further evidence that decreases in BK channel conductance induced by high glucose conditions are likely due to reduced *LRRC26* expression.

To determine the extent by which chronic elevation in glucose induces changes in ion channel function, we cultured NHBE cells in ALI media supplemented with either a 5.5 mM (normal) or 12.5 mM (high) glucose level throughout redifferentiation at the ALI. High glucose level did not significantly impact the transepithelial electrical resistance (TEER) of cultures (supplementary figure S1). However, BK channel activity was significantly reduced in NHBE cells cultured under high glucose levels compared to normal glucose (figures 2a and 2b). On the other hand, NHBE cells cultured under high glucose conditions showed a significant increase in CFTR conductance (figures 2d–2f), with a concomitant decrease in the activity of calcium-activated chloride channels (CaCCs) (supplementary figure S1). Expression of *CFTR* mRNA was correlated with elevated levels of glucose (supplementary figure S1). Despite this increase, the change in ASL volume over a 24-h period was not significantly different between NHBE cells cultured under high glucose compared to those cultured under normal glucose (figure 2c), likely due to the balance of changes in ion channel activities.

Hyperglycaemia induces the expression of inflammatory markers in NHBE cells in vitro

Hyperglycaemia can activate a pronounced inflammatory response [5], but whether it increases expression of inflammatory markers in the airway epithelium remains largely unknown. Compared to NHBE cells cultured under normal glucose conditions, NHBE cells cultured under high glucose conditions showed significant increases in the mRNA expression levels of a number of proinflammatory cytokines, including *IL-1β*, *IL-6* and *IL-8* (figures 3a–3c), as well as the inflammatory markers matrix metalloproteinase-9 (*MMP-9*) and transforming growth factor-β1 (*TGF-β1*) (figures 3d and 3e).

Hyperglycaemia induces BK channel dysfunction, loss of ASL volume and inflammation in CFBE cells in vitro

We next sought to determine the impact of high glucose conditions on BK channel function and ASL volume in primary CFBE cells *in vitro* (CFTR mutations of donor lungs are listed in supplementary table S2). CFBE cells cultured under high glucose conditions (24 h range: 10.1–12.5 mM) showed a significant reduction in BK channel function compared to CFBE cells cultured under normal glucose conditions (24 h range: 2.5–5.5 mM) (figures 4a and 4b and supplementary figure S2). The decrease in BK channel activity in CFBE cells also correlated with reduced *LRRC26* mRNA expression (figure 4c). Furthermore, CFBE cells under high glucose conditions showed significantly greater rates of ASL absorption over a 24 h period (figure 4d). *KCNMA1* mRNA expression was not affected by glucose levels (figure 4e), while ATP-stimulated K$^+$ currents in CFBE cells cultured under both normal and high glucose conditions were completely inhibited by the BK channel blocker paxilline (PAX) (figures 4g and 4h).

K$_{Ca}$3.1 potassium channels, which are encoded by the *KCNN4* gene, are also expressed at the apical membrane of bronchial epithelial cells and can be blocked by 1-((2-chlorophenyl)diphenylmethyl)-
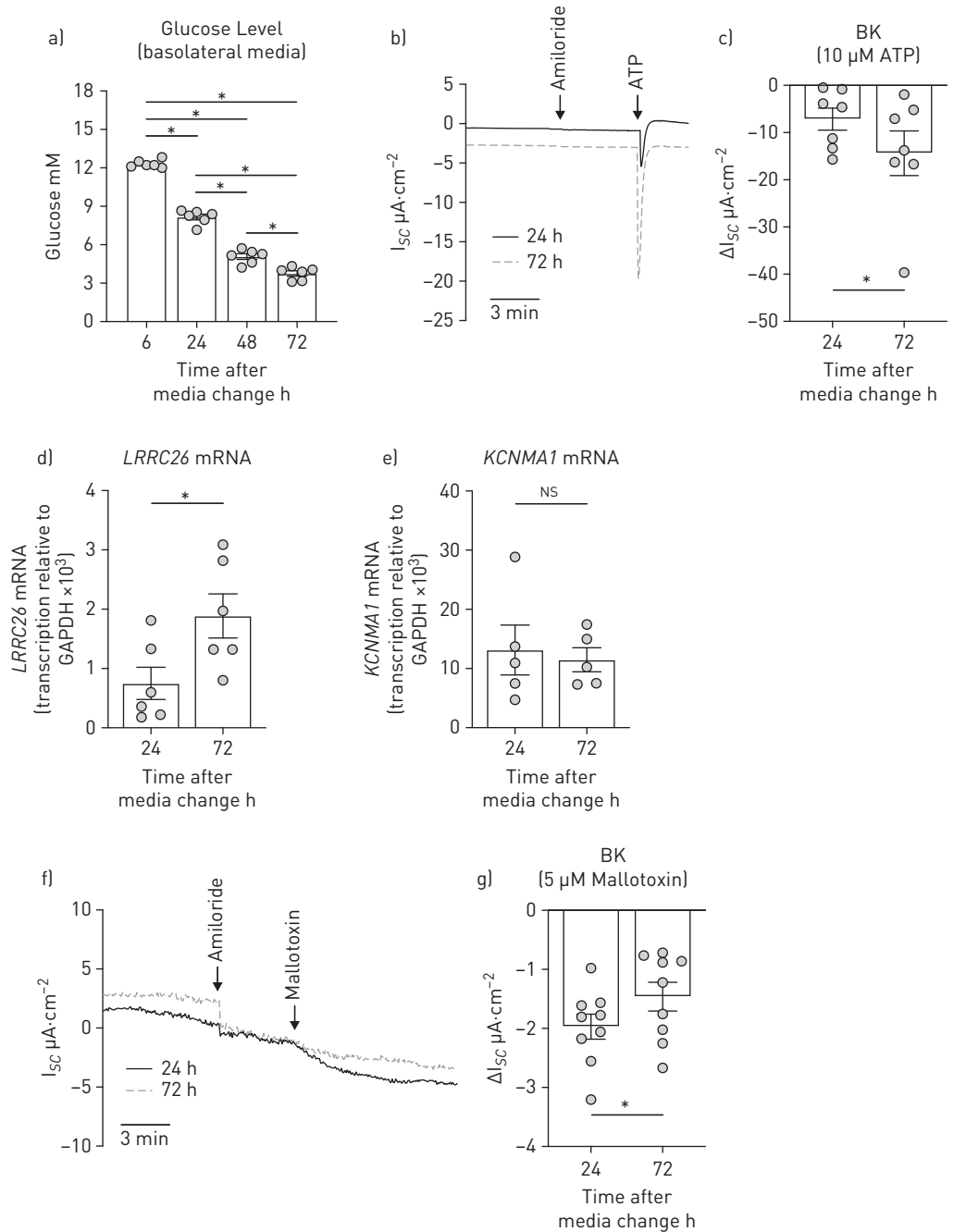
FIGURE 1 High glucose level negatively impacts large-conductance, Ca^{2+}-activated, voltage-dependent K^+ (BK) channel function in non-CF human bronchial epithelial (NHBE) cells *in vitro*. Panels are as follows: a) measurement of glucose levels in the basolateral media over time (n=6 lungs; $p < 0.05$, one-way ANOVA with Tukey's test). Fully differentiated NHBE cells were cultured in air–liquid interface (ALI) media with a high glucose level (12.5 mM or 225 mg·dL^{-1}); b) representative tracing of ATP-stimulated short-circuit current (I_{SC}) measured in an Ussing chamber with a basolateral to apical K^+ and apical to basolateral Na^+ gradient [27]. I_{SC} is a measure of net ionic current across the epithelium and thus is near zero at baseline; c) BK channel activity after media change (significantly lower at 24 h than at 72 h; n=7 lungs; $p < 0.05$, one-tailed t-test); d) mRNA expression levels of *LRRC26* on media change (inverse correlation with glucose levels; n=6 from three lungs; $p < 0.05$, unpaired t-test); e) mRNA expression levels of *KCNMA1* on media change (no significant difference in NHBE cells at 24 h and 72 h after media change; n=6 lungs); f) representative tracing of mallotoxin-stimulated I_{SC} measured in an Ussing chamber with a basolateral to apical K^+ gradient [13]; g) BK channel activity (mallotoxin activation) on media change (significantly greater in NHBE cells 24 h after media change compared to 72 h; n=9 from four lungs; $p < 0.05$, Student's t-test). These data indicate that KCNMA1 is still at the plasma membrane and opens with mallotoxin when leucine-rich repeat-containing protein 26 (LRRC26) associations are reduced [13]. Data are shown as mean±SEM. GAPDH: glyceraldehyde 3-phosphate dehydrogenase; NS: not significant; SEM: standard error of the mean. *: $p < 0.05$.
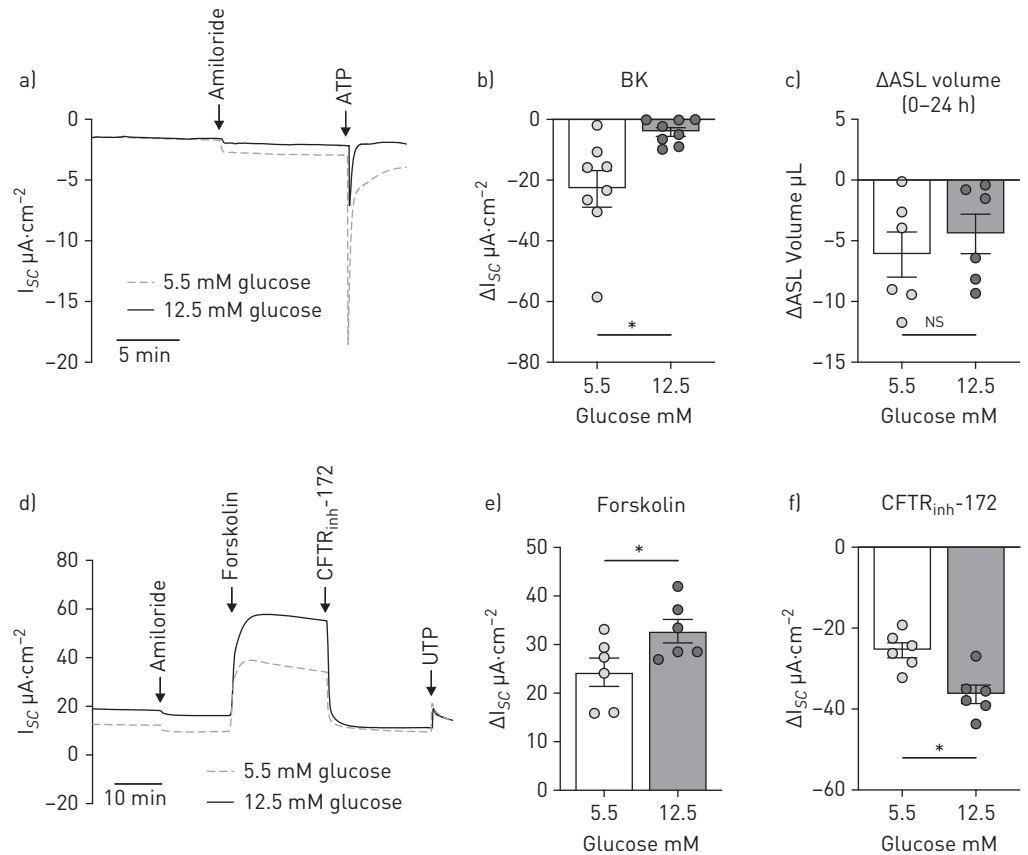
FIGURE 2 High glucose level differentially affects ion channel function in non-CF human bronchial epithelial (NHBE) cells *in vitro*. Panels are as follows: a) representative tracing of ATP-stimulated short-circuit current (I_{SC}) with a basolateral to apical K^+ gradient [27]. Fully differentiated NHBE cells were cultured in air–liquid interface (ALI) media with starting glucose concentrations of 5.5 mM or 12.5 mM. Ussing chamber experiments were performed on NHBE cells 20–24 h after basolateral media change; b) large-conductance, Ca^{2+}-activated, voltage-dependent K^+ (BK) channel activity by glucose level. Activity is significantly decreased in NHBE cells cultured under high glucose (n=8 from six lungs); c) change in airway surface liquid (ASL) volume over a 24 h period by glucose level. Change did not differ significantly between NHBE cells cultured under 5.5 mM or 12.5 mM glucose (n=6 from three lungs); d) representative tracing of I_{SC} following forskolin stimulation and CF transmembrane conductance regulator (CFTR) inhibition by CFTR$_{inh}$-172, from fully differentiated NHBE cells cultured in ALI media with 5.5 mM or 12.5 mM glucose. Ussing chamber experiments were performed on NHBE cells 20–24 h after basolateral media change; e) change in CFTR current on forskolin usage by glucose level. Stimulation is significantly increased in NHBE cells cultured under high glucose (n=6 lungs); f) change in CFTR current on CFTR$_{inh}$-172 usage by glucose level. Inhibition is significantly increased in NHBE cells cultured under high glucose (n=6 lungs). Data are shown as mean±SEM. UTP: uridine-5′-triphosphate; SEM: standard error of the mean. *: $p<0.05$ (by t-test).

1*H*-pyrazole (TRAM-34) [29]. Glucose levels did not impact *KCNN4* mRNA expression in CFBE cells (figure 4f). Furthermore, while TRAM-34 had no effect on ATP-stimulated K^+ currents in CFBE cells cultured under normal glucose conditions (figure 4g), such currents were reduced by TRAM-34 in CFBE cells cultured under high glucose conditions, although the decrease was not significant (figure 4h).

High glucose conditions did not lead to a significant increase in the expression of *IL-1*β, *IL-8*, or *TGF-β1* mRNA in CFBE cells (supplementary figure S3). On the other hand, CFBE cells cultured under high glucose conditions did show significant increases in the mRNA expression of *MMP-9* and cyclooxygenase-2 (*COX-2*) compared to CFBE cells cultured under normal glucose media (figures 5a and 5b). AGE are formed during hyperglycaemia and bind to the pro-inflammatory RAGE [14]. Interestingly, CFBE cells under high glucose conditions demonstrated a significant increase in the expression of *RAGE* mRNA compared to CFBE cells under normal glucose conditions (figure 5c). A similar increase in *RAGE* mRNA expression was not observed in NHBE cells in response to high glucose conditions (figure 3f). We also detected a significant increase in secreted IL-1β and IL-8 protein in the basolateral media of CFBE cells cultured under high glucose conditions compared to CFBE cells cultured under normal glucose conditions (figures 5d and 5e). Levels of IL-6 in the basolateral media remained unchanged (figure 5e).
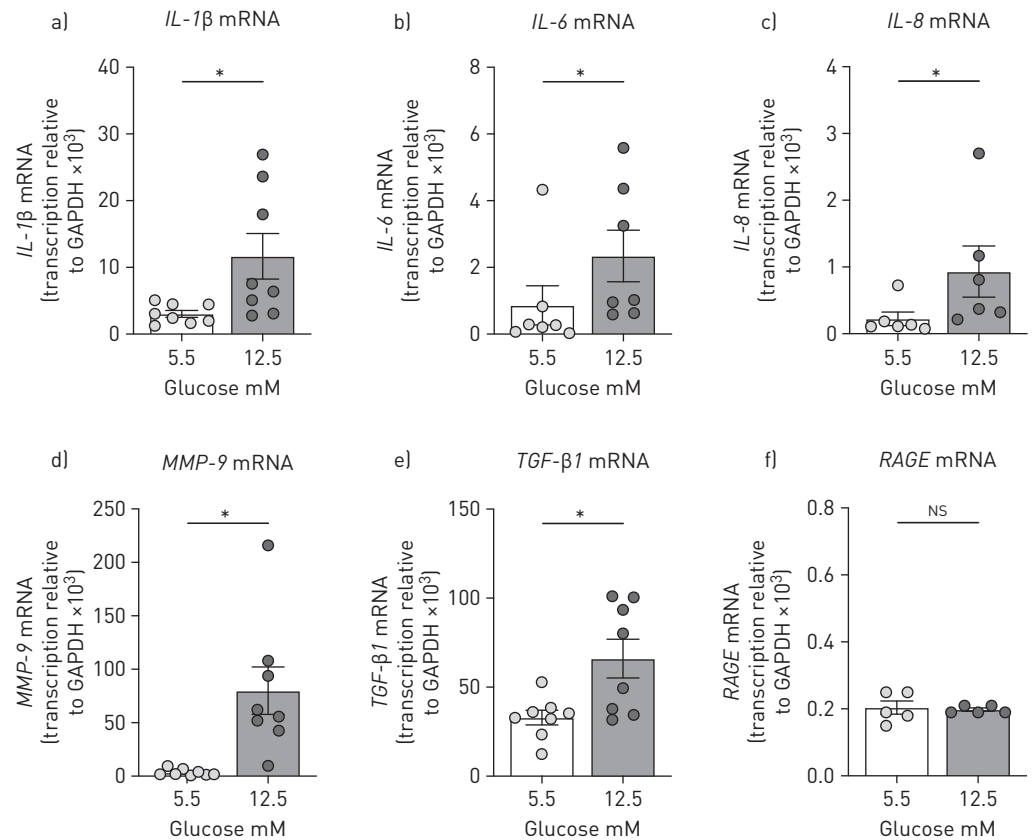
FIGURE 3 High glucose induces expression of inflammatory markers in non-CF human bronchial epithelial (NHBE) cells *in vitro*. NHBE cells cultured under high glucose show significant increases in mRNA expression levels of a) *IL-1*β, b) *IL-6*, c) *IL-8*, d) *MMP-9* and (e) *TGF-β1* compared to NHBE cells cultured under normal glucose (n⩾6 from six lungs). However, high glucose does not affect the expression of *RAGE* mRNA in NHBE cells (n=5 from four lungs) (f). Data are shown as mean±SEM. IL: interleukin; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; MMP-9: matrix metalloproteinase-9; TGF-β: transforming growth factor-β; RAGE: receptor for advanced glycation endproducts; NS: not significant; SEM: standard error of the mean. *: $p<0.05$ (by Wilcoxon test or t-test).

The RAGE agonist HMGB1 induces BK channel dysfunction and ASL volume loss in CFBE cells in vitro

It is possible that the increase in *RAGE* expression in CFBE cells is caused by BK channel dysfunction induced by hyperglycaemia. To address this possibility, we treated CFBE cells cultured under normal (5.5 mM) glucose conditions with recombinant HMGB1 protein and measured BK channel function. HMGB1 is a RAGE ligand and marker of airway epithelial injury that induces a strong pro-inflammatory response [30–32]. CFBE cells treated with HMGB1 (100 ng·mL^{-1}) in the basolateral media for 24 h showed a significant reduction in BK channel function compared to controls (figures 6a and 6b). The reduction in BK channel activity correlated with increased ASL absorption (figure 6c). These data suggest that RAGE activation is upstream of BK channel dysfunction.

CGM variables for hyperglycaemia correlate with decreased LRRC26 expression in those with CF and CFRD

Based on our *in vitro* data, we initiated an observational study to determine whether there was a correlation between glycaemic excursions (as measured by CGM) and *LRRC26* expression in the upper airways of CF patients. A total of 21 participants (CF (n=6) and CFRD (n=15)) were enrolled in the study (see supplementary table S3 for baseline participant characteristics). Four subjects (CF (n=1) and CFRD (n=3)) were excluded from analysis due to monitor malfunction, monitor use less than 5 days, or inadequate mRNA in nasal brush samples. The CFRD group was older (mean age: 32 years *versus* 22 years; 95% CI of difference between groups 5.3–14.7) and had lower baseline lung function (mean % predicted forced expiratory volume in 1 s (FEV$_1$): 64% *versus* 98%; 95% CI of difference between groups −50.77 to −17.23) as compared to the CF group without diabetes. There were no differences in *CFTR* mutation group (F508del homozygous or heterozygote/minimum function) or CFTR modulator use
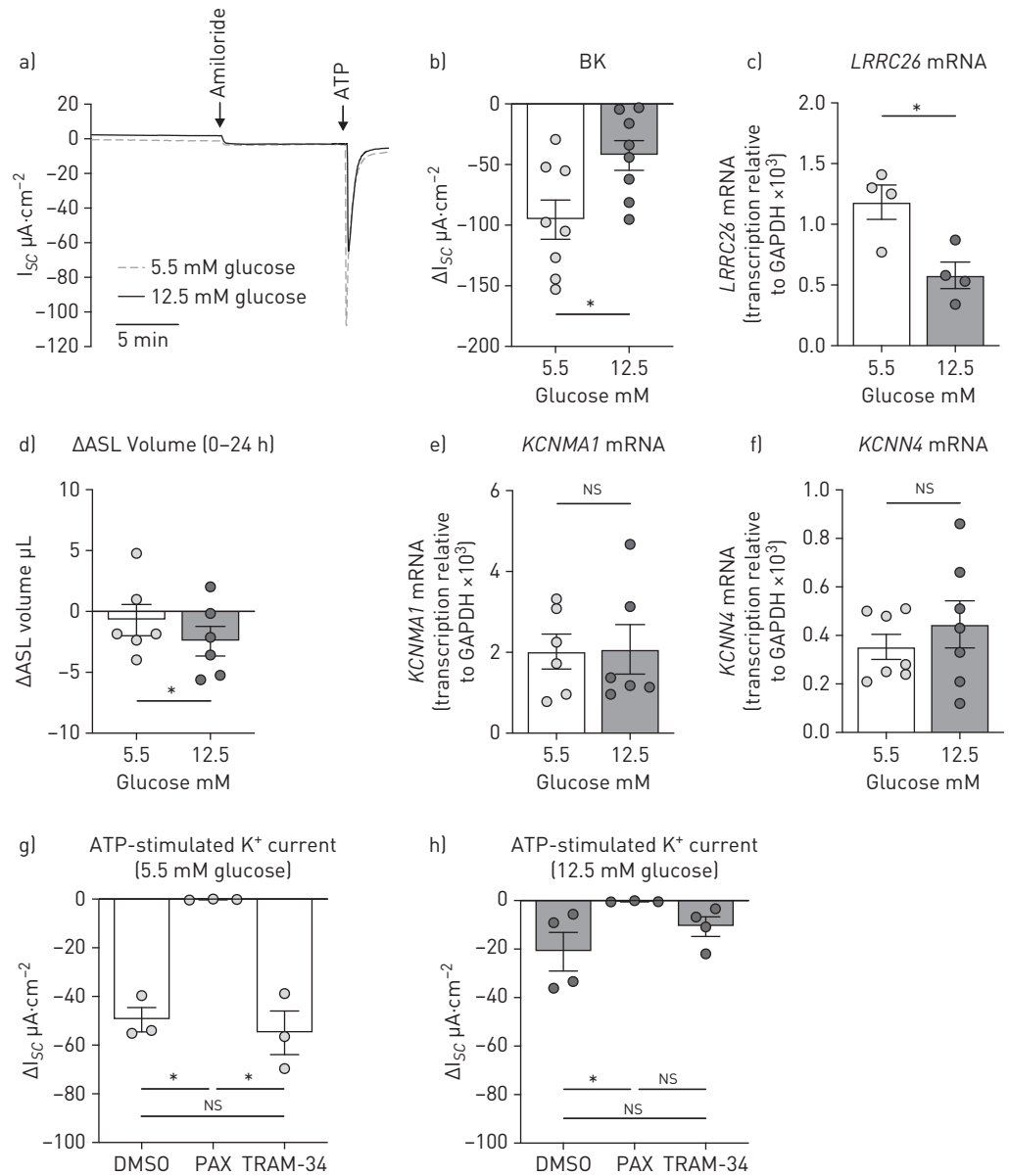
FIGURE 4 High glucose reduces large-conductance, Ca^{2+}-activated, voltage-dependent K^+ (BK) channel function and leads to a loss of airway surface liquid (ASL) volume in CF bronchial epithelial (CFBE) cells *in vitro*. Panels are as follows: a) representative tracing of ATP-stimulated short-circuit current (I_{SC}) with a basolateral to apical K^+ and apical to basolateral Na^+ gradient [27]. Fully differentiated CFBE cells were cultured in air–liquid interface (ALI) media with starting glucose concentrations of 5.5 mM or 12.5 mM. Ussing chamber experiments were performed on CFBE cells 20–24 h after basolateral media change; b) BK channel activity by glucose level. Activity is significantly decreased in CFBE cells cultured under high glucose (24 h range: 10.1–12.5 mM) compared to normal glucose (24 h range: 2.5–5.5 mM) (n=8 from seven CF lungs). Significance determined by t-test; c) expression of *LRRC26* mRNA by glucose level. Expression inversely correlates with glucose level (n=4 CF lungs). Significance determined by t-test; d) ASL absorption by glucose level. CFBE cells cultured under high glucose show increased ASL absorption (as indicated by a more negative ΔASL volume) over a 24 h period compared to cells cultured under normal glucose (n=6 CF lungs). Significance determined by t-test; e) expression of *KCNMA1* mRNA by glucose level. Expression is not significantly different in CFBE cells cultured in ALI media with starting glucose concentrations of either 5.5 mM or 12.5 mM (n=6 from six CF lungs). Significance determined by Wilcoxon test; f) expression of *KCNN4* mRNA (*KCNN4* encodes the $K_{Ca}3.1$ potassium channel) by glucose level. High glucose does not affect the expression of *KCNN4* mRNA in CFBE cells (n=7 from seven CF lungs). Significance determined by Wilcoxon test; g) ATP-stimulated K^+ currents in CFBE cells cultured in ALI media (with a starting glucose concentration of 5 mM) are significantly reduced by 10 µM paxilline (PAX) but not by 1 µM TRAM-34 (1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole) (n=3 CF lungs). Significance determined by one-way ANOVA with Tukey's test; h) ATP-stimulated K^+ currents in CFBE cells cultured in ALI media (with a starting glucose concentration of 12.5 mM) are significantly reduced by 10 µM PAX; however, the reduction by TRAM-34 was not significant (n⩾3 CF lungs). Significance determined by Kruskal–Wallis test with Dunn's test. Data are shown as mean±SEM. LRRC26: leucine-rich repeat-containing protein 26; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; DMSO: dimethyl sulfoxide; NS: not significant; SEM: standard error of the mean. *: p<0.05.
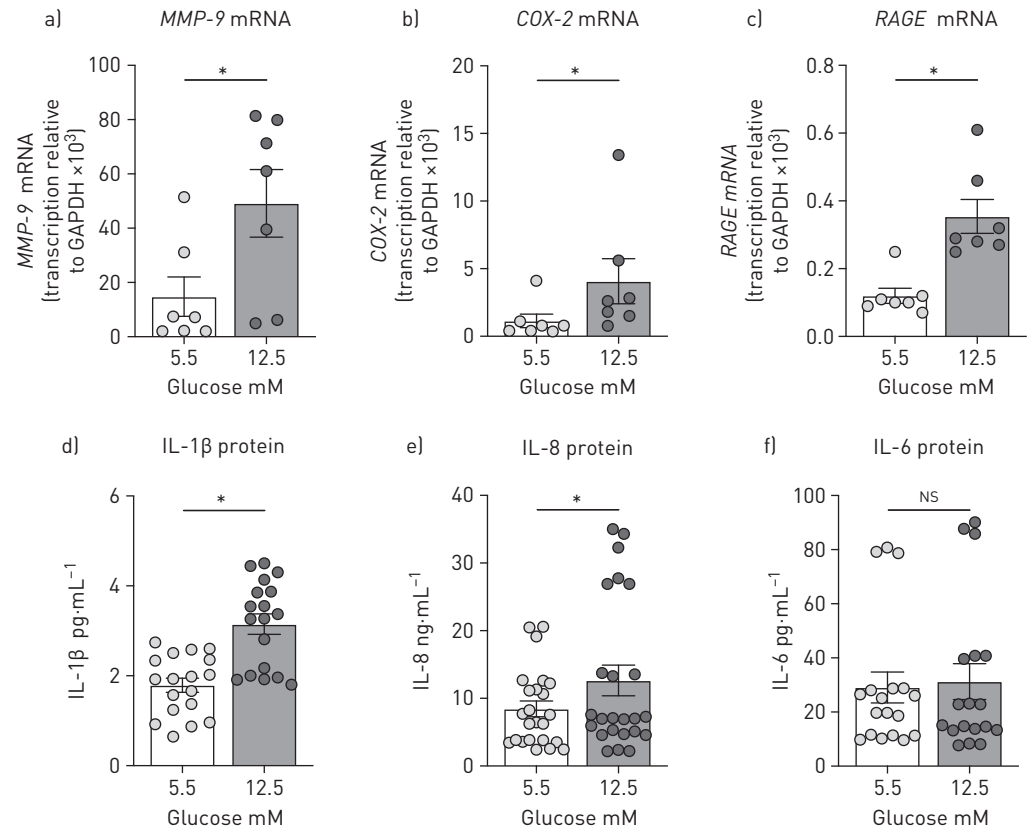
FIGURE 5 High glucose induces expression of inflammation markers and receptors for advanced glycation endproducts (RAGE) in CF bronchial epithelial (CFBE) cells *in vitro*. CFBE cells cultured under high glucose show significant increases in mRNA expression levels of a) *MMP-9*, b) *COX-2* and c) *RAGE* compared to CFBE cells cultured under normal glucose conditions (n=7 CF lungs). CFBE cells cultured under high glucose show a significant increase in expression levels of d) IL-1β protein (n=18, triplicates from six CF lungs) and e) IL-8 protein (n=24, triplicates from six CF lungs) in the basolateral media, as determined by enzyme-linked immunosorbent assay (ELISA). Expression levels of IL-6 protein f) are not significantly different in the basolateral media of CFBE cells cultured under normal *versus* high glucose (n=18, triplicates from six CF lungs). Data are shown as mean±SEM. MMP-9: matrix metalloproteinase-9; COX-2: cyclooxygenase-2; IL: interleukin; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; NS: not significant; SEM: standard error of the mean. *: $p < 0.05$ (by Wilcoxon test).

between groups. CGM variables of interest were uniformly higher in the CFRD group as compared to the CF group (supplementary table S4).

A significant, inverse correlation was found between *LRRC26* expression in nasal cells and percentage time of glucose >140 mg·dL^{-1} (r= −0.51, p=0.03), percentage time glucose >200 mg·dL^{-1} (r= −0.54, p=0.02) and AUC for glucose >180 mg·dL^{-1} (r= −0.48, p=0.04) (figures 7a–7c). Other CGM variables of interest, including GMI (r= −0.46, p=0.06) and MAGE (r= −0.35, p=0.17), demonstrated a negative trend with *LRRC26* expression but did not achieve significance (figures 7d and 7e). There was no significant association between age and *LRRC26* expression (or a difference in *LRRC26* expression) between female and male sex or CF and CFRD (supplementary figure S4). A significant correlation was found in the CFRD group between *RAGE* expression and CGM variables including percentage time glucose >140 mg·dL^{-1} and MAGE (p<0.05) (figures 8a and 8b). Other CGM variables of interest also demonstrated positive trends but failed to reach significance. As this small sample size is sensitive to outliers, excluding the participant with the greatest *RAGE* expression resulted in a significant association between *RAGE* expression and AUC for glucose >180 mg·dL^{-1}, percentage time glucose >200 mg·dL^{-1} and GMI (p<0.05) (figures 7c–7e).

Discussion

CFRD is a common extrapulmonary comorbidity in adults with CF and is associated with increased lung function decline [1, 2]. However, the mechanisms underlying this association are unknown. In this study,
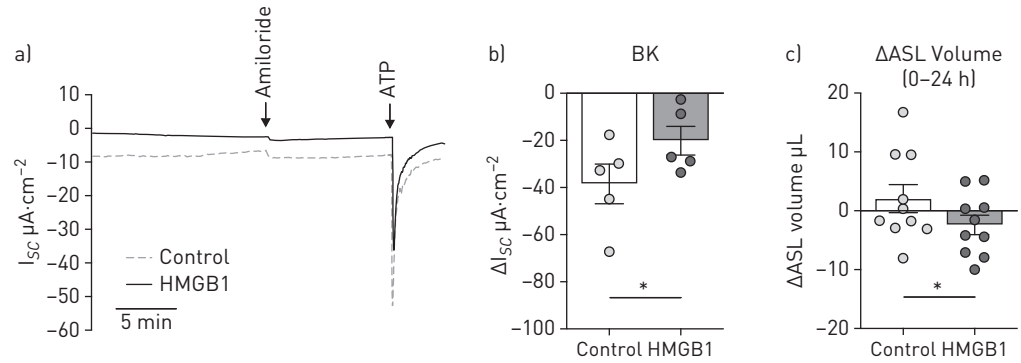
FIGURE 6 High mobility group box 1 (HMGB1) protein reduces large-conductance, Ca^{2+}-activated, voltage-dependent K$^+$ (BK) channel function and leads to a loss of airway surface liquid (ASL) volume in CF bronchial epithelial (CFBE) cells *in vitro*. Panels are as follows: a) representative tracing of ATP-stimulated short-circuit current (I$_{SC}$) with a basolateral to apical K$^+$ gradient and apical to basolateral Na$^+$ gradient [27]. Fully differentiated CFBE cells were cultured in air–liquid interface (ALI) media with a starting glucose concentration of 5.5 mM, treated for 24 h with recombinant HMGB1 (100 ng·mL^{-1}) or control vehicle (water); b) BK channel activity is significantly decreased in CFBE cells treated with recombinant HMGB1 (n=5 from four CF lungs); c) CFBE cells treated with recombinant HMGB1 show increased ASL absorption (as indicated by a more negative ΔASL volume) over 24 h. Data are shown as mean±standard error of the mean (SEM). *: p<0.05 (by t-test).

we demonstrated in an *in vitro* model of the CF airway epithelia that hyperglycaemia leads to impaired BK channel function, decreased ASL volume and elevation in the expression of inflammatory markers. This finding was confirmed *in vivo* where *LRRC26* expression, which predicts BK channel function in airway epithelia [13, 27, 33, 34], was inversely correlated with CGM variables for hyperglycaemia in study participants with CF and CFRD.

BK channels are likely to be the primary apically expressed K$^+$ channels in the airway epithelium [13, 16]. BK channel function is critical for ASL hydration since inhibition, either by knockdown of the pore-forming α-subunit KCNMA1 or of LRRC26, causes a significant reduction in ASL volume, even in the presence of fully functional CFTR [16]. Interestingly, although high glucose conditions correlated with reduced BK channel function in NHBE cells, ASL volumes were largely unaffected. This was likely due to the unexpected increase in CFTR conductance in these cells, suggesting that the airway epithelium employs mechanisms that differentially affect ion channel function to maintain ASL homeostasis during hyperglycaemic excursions. Although a previous study found that hyperglycaemia leads to a reduction in CFTR conductance, this study was conducted at higher glucose concentration (25 mM) in CFBE cell monolayers transduced with wild-type CFTR and may not necessarily reflect endogenous CFTR function in NHBE cells [12]. When CFTR function is limited or absent, as in CF, the airway epithelium is unlikely to maintain ASL homeostasis when glucose levels become elevated. Indeed, we found increased rates of ASL absorption in CFBE cells challenged with high glucose levels that correlated with reduced BK channel function and *LRRC26* expression.

It is likely that hyperglycaemia also affects other ion channels that are important for ASL hydration. For example, activation of K$_{Ca}$3.1 channels, which are also expressed at the apical membrane, is thought to increase chloride secretion and improve airway hydration [28, 35]. Although high glucose levels did not have a significant impact on the expression or function of K$_{Ca}$3.1 channels in our experiments, we cannot completely rule out the possibility that they contribute to hyperglycaemia-induced changes in mucociliary clearance. Furthermore, our data in NHBE cells show that uridine-5'-triphosphate (UTP)-stimulated Cl$^-$ currents are impacted by high glucose levels. However, these studies provide some of the first *in vitro* evidence that high glucose conditions can directly impact the expression and function of an ion channel known to be critical for mucociliary clearance in the CF airways.

The underlying link between BK channel dysfunction and hyperglycaemia is unclear, but we hypothesise that it is related to inflammation. In hyperglycaemia the AGE–RAGE signalling axis is upregulated and this contributes to excess inflammation [14]. We found that CFBE cells under high glucose conditions have significantly elevated levels of *RAGE* mRNA expression. Interestingly, we did not see a similar increase in *RAGE* expression in NHBE cells under high glucose conditions. This is consistent with previous reports that showed significantly higher levels of *RAGE* expression in the sputum of CF and CFRD subjects compared to non-CF diabetic subjects [11]. Indeed, levels of RAGE and RAGE ligands have been found to be increased in CF airways and levels of AGE have been shown to negatively correlate
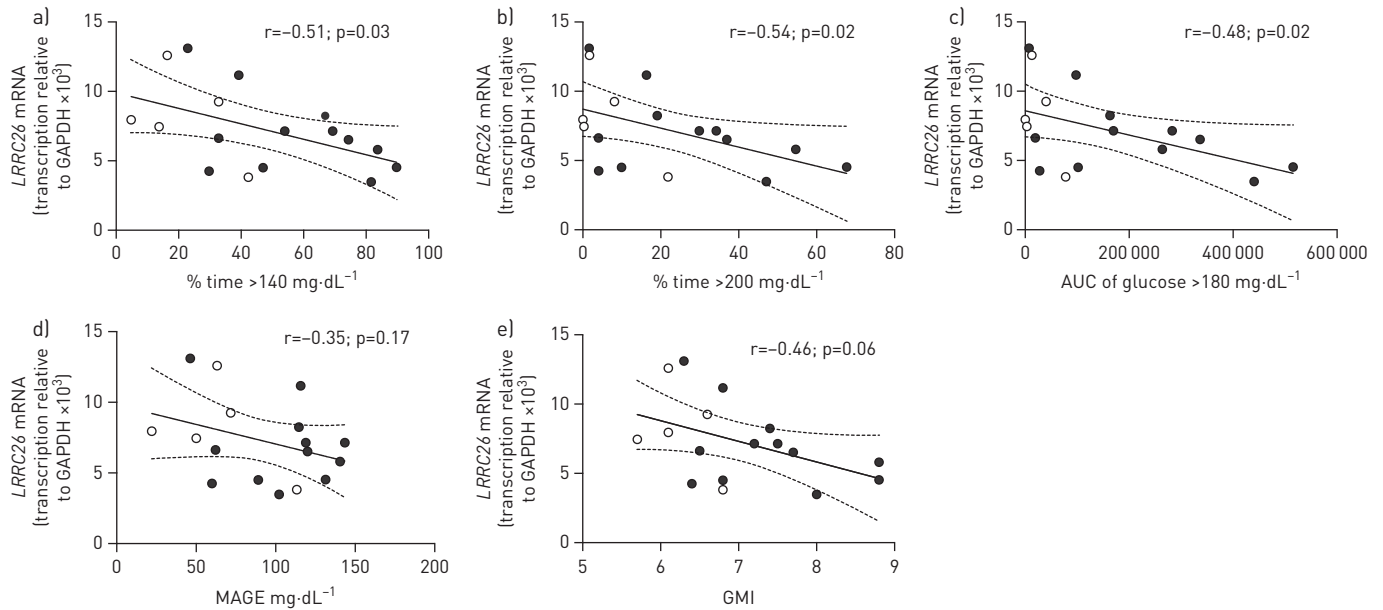
FIGURE 7 Hyperglycaemic excursions correlate with decreased *LRRC26* mRNA expression in participants with cystic fibrosis (CF) and CF-related diabetes mellitus (CFRD). Inverse correlation is observed between nasal cell *LRRC26* expression in CF (n=5, open circles) and CFRD (n=12, shaded circles) and the continuous glucose monitoring (CGM) variables a) percentage time glucose >140 mg·dL^{-1}, b) percentage time glucose >200 mg·dL^{-1} and c) average area under the curve (AUC) of glucose >180 mg·dL^{-1} (p<0.05, by Pearson's correlation coefficient). In addition, there was an inverse trend between *LRRC26* expression and the CGM variables d) mean amplitude of glycaemic excursion (MAGE) and e) glucose management indicator (GMI); however, it did not reach statistical significance (p>0.05, by Pearson's correlation coefficient). Graphs include a trend line (solid) and 95% confidence interval (CI) lines (dashed). LRRC26: leucine-rich repeat-containing protein 26; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

with % predicted FEV$_1$ [10, 11]. Furthermore, levels of HMGB1 are significantly elevated in the sputum and serum of CF patients and particularly at the onset of CFRD [33]. The increase in IL-1β secretion in CFBE cells in response to high glucose levels is also particularly interesting in light of a recent study demonstrating the importance of IL-1β in promoting mucus hypersecretion and increasing the expression of proinflammatory mediators in CF airways in the absence of infection [36]. Levels of *LRRC26* expression
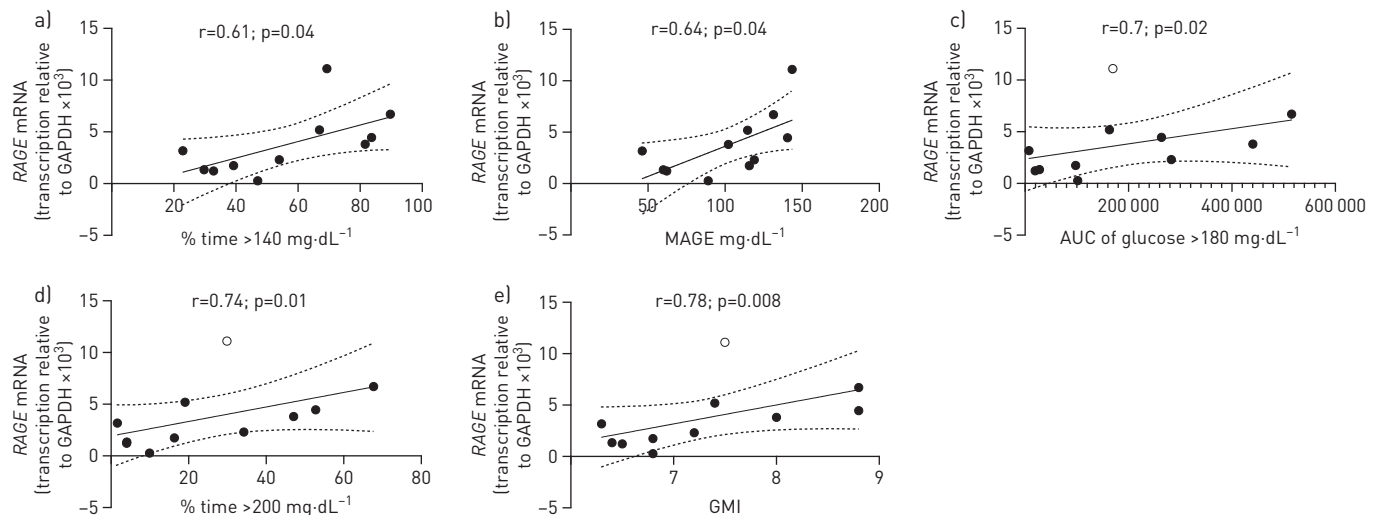
FIGURE 8 *RAGE* expression is correlated with hyperglycaemic excursions in CF-related diabetes mellitus (CFRD). In participants with CFRD, correlation is observed between nasal cell *RAGE* expression and the continuous glucose monitoring (CGM) variables (a) percentage time glucose >140 mg·dL^{-1} and (b) mean amplitude of glycaemic excursion (MAGE) (p<0.05, by Pearson's correlation coefficient). After exclusion of an outlier (open circle), there is also a significant correlation between *RAGE* expression and the CGM variables (c) area under the curve (AUC) of glucose >180 mg·dL^{-1}, (d) percentage time glucose >200 mg·dL^{-1} and (e) glucose management indicator (GMI) (p<0.05, by Pearson's correlation coefficient). Graphs include a trend line (solid) and 95% confidence interval (CI) lines (dashed). RAGE: receptor for advanced glycation endproducts; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.
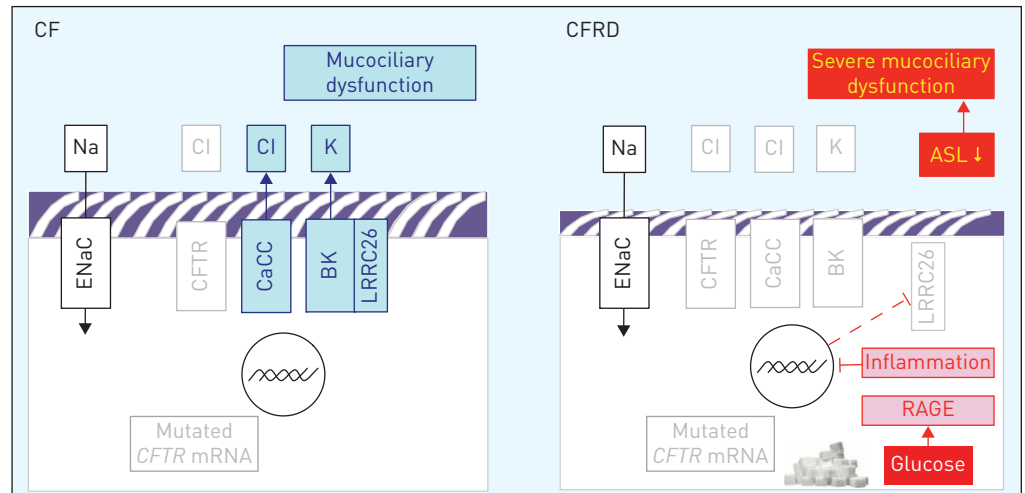
FIGURE 9 Schematic of the effects of hyperglycaemia on airway cells in cystic fibrosis (CF). Increased glucose (sugar) leads to receptor for advanced glycation endproduct (RAGE) activation with inflammation and a decrease in leucine-rich repeat-containing protein 26 (LRRC26) expression, which decreases large-conductance, Ca^{2+}-activated, voltage-dependent K^+ (BK) channel function (and concomitant calcium-activated chloride channel (CaCC) function). CFRD: CF-related diabetes mellitus; ASL: airway surface liquid; CFTR: CF transmembrane conductance regulator; ENaC: epithelial sodium channel.

are sensitive to inflammation in CFBE cells [33]. Thus, future studies will focus on how RAGE activation and possibly Il-1β may potentially regulate BK channel function.

Based on these *in vitro* data, we initiated a clinical study to determine whether *LRRC26* expression is impacted by variations in glucose in CF patients. Interestingly, there was no significant difference in *LRRC26* expression between CF and CFRD patients. Instead, the inverse correlation with *LRRC26* expression occurred across a spectrum of glycaemic control in both groups. This suggests glycaemic control is an important factor in both CF and CFRD. Also, a correlation between *RAGE* expression and hyperglycaemia was observed in participants with CFRD, mirroring the *in vitro* results. However, there were several limitations to our study. First, we relied on historical OGTT to define CF and CFRD, which could have led to incorrect categorisation of participants. However, as our results suggest that level of glycaemic control was more strongly associated with *LRRC26* expression than CFRD status, this was not likely to affect our findings. Secondly, a nonsignificant but noticeable discrepancy in CGM sampling periods between the two groups was observed. While the CGM variables are means and proportions, which are less likely to be affected by differences in sampling length, this difference could lead to misestimation of true glycaemic control. Thirdly, while CFTR modulator use was not found to be associated with *LRRC26* expression, we were not able to compare differences between specific CFTR modulators given our relatively small number of subjects. It should be noted that our aims were exploratory and no corrections were made for multiple comparisons given the small sample size.

Importantly, we believe these findings remain relevant in the context of highly effective CFTR modulator therapy. Previous observational studies have demonstrated that there are improvements in insulin secretion after initiation of highly effective CFTR modulator therapy. However, their potential effects on hyperglycaemic excursions are less well known [37, 38]. A study of subjects on ivacaftor showed that, while there was an increase in insulin after 16 weeks of ivacaftor use, there was no significant improvement in peak glucose or AUC for glucose during a mixed meal tolerance test [39]. While this study was small in size and subjects had mostly normal OGTTs prior to ivacaftor use, it highlights that glycaemic excursions may persist in those receiving highly effective modulators. Furthermore, impaired pancreatic function and glycaemic abnormalities are present at birth in the CF ferret model and are prevalent in infants and young children with CF, suggesting earlier interventions are needed to preserve lung function in CFRD [40, 41]. Thus, there is a need for continued investigation into the effects of hyperglycaemia on pulmonary function decline in CF.

In conclusion and summarised in figure 9, we have demonstrated that hyperglycaemia increases inflammation with a detrimental effect on *LRRC26* expression and therefore impairment of BK channel function. To our knowledge this is the first study to provide a connection between hyperglycaemic excursions and worse parameters important for mucociliary function in CF. Further mechanistic and interventional studies are needed to define therapies that will improve the disparity in outcomes between CF and CFRD.

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