Interaction with CREB binding protein modulates the activities of Nrf2 and NF-κB in cystic fibrosis airway epithelial cells

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Ziady AG, Sokolow A, Shank S, Corey D, Myers R, Plafker S, Kelley TJ. Interaction with CREB binding protein modulates the activities of Nrf2 and NF-κB in cystic fibrosis airway epithelial cells. Am J Physiol Lung Cell Mol Physiol 302: L1221–L1231, 2012.—Cystic fibrosis (CF) is characterized by inflammatory lung disease that significantly contributes to morbidity and mortality. Airway epithelial cells play a role in the inflammatory signaling in CF and have been reported to exhibit a number of dysfunctions in signaling cascades that modulate inflammation. Previously, we reported that the activity of nuclear factor erythroid-derived like 2 (Nrf2), a transcription factor that regulates antioxidant and cytoprotective protein expression, is diminished in CF epithelia (4). In this report, we examined the mechanism of Nrf2 dysregulation in vitro in human airway epithelial cell lines and primary cells and in vivo in nasal epithelia excised from ΔF508 CF mutant mice. We found that cAMP-mediated signaling markedly reduces Nrf2 activity in CF vs. non-CF cells. Rp-cAMPS, a cAMP competitor, significantly corrected Nrf2 activity in CF cells, predominantly by increasing the nuclear accumulation of the transcription factor. Furthermore, we found that Rp-cAMPS significantly decreased NF-κB activation following inflammatory stimulation of CF cells. Further investigation revealed that Nrf2 and NF-κB compete for the transcriptional coactivator CREB responsive element-binding protein (CREB) binding protein (CBP) and that Rp-cAMPS shifts CBP association in favor of Nrf2. Thus our findings provide a link between feedback to CF transmembrane regulator dysfunction and dysregulation of an inflammatory signaling pathway that modulates the coordinated activities of Nrf2 and NF-κB. Furthermore, our studies suggest that strategies that shift CBP association away from NF-κB and toward Nrf2 could have potential therapeutic efficacy for reducing inflammation in patients with CF.

H2O2-mediated inflammatory signaling; airway epithelia; cAMP responsive element-binding protein

IN ADDITION TO DYSFUNCTION of the cystic fibrosis (CF) transmembrane regulator (CFTR) chloride channel, CF airway epithelia are characterized by a number of secondary defects including abnormal epithelial sodium channel function (15) and inflammatory signaling (5,30). Excessive inflammatory responses have been reported in patients with CF (29) and CF mice (11). Furthermore, there is evidence that resolution of inflammation in CF is impaired (17,27). Some studies have demonstrated that inflammation is not innate to CF (2). Nevertheless, it is widely accepted that inflammation diminishes lung function and leads to mortality and that controlling inflammation slows lung deterioration (16). Despite the significance of inflammation in CF disease, the molecular underpinnings linking CFTR dysfunction and inflammatory signaling remain to be established.

A potential mechanism for excessive inflammation in CF is the activation of NF-κB by oxidative stress, which results from an imbalance of oxidants (i.e., H2O2) and antioxidants (i.e., glutathione) in the cell (9,32,34). Redox signaling pathways have been shown to play a key role in the activation of inflammatory cascades in the lung (37,44). For example, hydrogen peroxide (H2O2) has been reported to mediate IL-1β (18) and TNF-α (8,20) receptor signaling cascades. Furthermore, a number of studies have demonstrated that H2O2 directly activates NF-κB (25,36). Antioxidants, which reduce H2O2 levels, inhibit the production of cytokines following the stimulation of the IL-1β and TNF-α receptors (7,8,18), indicating an essential role of oxidants in the propagation of normal inflammatory signaling. We previously reported that intracellular steady-state levels of H2O2 were elevated in CF model primary and immortalized epithelia and that this elevation in H2O2 promoted hyperinflammatory responses (47).

A key modulator of redox balance and signaling is the transcription factor, nuclear-factor-E2-related factor 2 (Nrf2), which drives the expression of a battery of genes that protect against electrophilic and oxidative stress (reviewed in Ref. 45). It plays a central role in the cellular antioxidant defense system by inducing the expression of detoxifying and antioxidant factors that maintain and restore redox balance. During homeostasis, Nrf2 is sequestered in the cytoplasm by its interaction with Kelch-like erythroid cell-derived protein with cap’n’collar homology-associated protein 1 (Keap1) (28). Keap1 is a substrate adaptor that recruits Nrf2 to a cullin-3-based ubiquitin ligase for subsequent ubiquitin modification. Increased oxidant load in cells results in the oxidation of cysteine residues on Keap1 and dissociation of the Keap1:Nrf2 complex from the cullin-3 scaffold (46). Nrf2 is subsequently translocated to the nucleus where it binds to the antioxidant response elements (AREs) in the promoters of its target genes (4,24). After the reestablishment of redox homeostasis, Nrf2 activity is terminated, but a complete mechanistic understanding of how this happens is lacking. Recent evidence suggests that Keap1 is chaperoned to the nucleus by prothymosin (PTM)-α, where interaction with PTM-α is exchanged for the binding of Nrf2 (33). The Keap1-Nrf2 complex is then exported from the nucleus and reassembled with the cytoplasmic cullin-3-based ligase, and Nrf2 is ubiquitinated and targeted for degradation. Removal from the nucleus serves as one mechanism to limit the transcriptional activity of Nrf2. Another level of control is

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modulation of the availability of transcription coactivators in the nucleus, such as cAMP responsive element-binding protein (CREB) binding protein (CBP), which has been shown to interact with and activate Nrf2 (13).

Previously, we demonstrated that steady-state intracellular \( \text{H}_2\text{O}_2 \) is significantly elevated in CF epithelial cells (7). However, despite this increase in \( \text{H}_2\text{O}_2 \), Nrf2 activity and the expression of a number of antioxidant proteins were significantly diminished in CF epithelia, both in culture and in vivo (7). This response is paradoxical because increased steady \( \text{H}_2\text{O}_2 \) is expected to promote Nrf2 activation and increase the expression of Nrf2 target genes. Therefore, we hypothesized that the suppression of Nrf2 activity in CF epithelia may be mediated by the modulation of one or more Nrf2 binding partners in the nucleus, following dissociation of the transcription factor from Keap1. This hypothesis is consistent with our earlier studies showing that the absence of CFTR function results in the alteration of cAMP-mediated signaling and phosphorylation of CREB (26). Together, these findings indicate that cAMP-mediated regulation of CBP may be a nexus for modulating the relative activities of critical proinflammatory and cytoprotective transcription factors such as NF-kB and Nrf2.

Here, we report that the cAMP-binding competitor Rp-cAMPS significantly rescues Nrf2 activity in human CF epithelial cell lines and primary bronchial epithelia. Using an ARE-driven luciferase reporter assay, we found that Rp-cAMPS significantly increased Nrf2 activity in CF epithelia. This increase in Nrf2 activity was sufficient to rectify the decrease in Nrf2 target gene expression and results in a reduction of the elevated intracellular \( \text{H}_2\text{O}_2 \) and cytokine production by CF cells. We explored the mechanism of Nrf2 activation by Rp-cAMPS and found that treatment with the compound increased the nuclear fraction of Nrf2 in CF cells to non-CF control levels by increasing the interaction with CBP of Nrf2. Furthermore, the Rp-cAMPS-mediated increase in Nrf2-CBP association is concurrent with a decrease in CBP interaction with NF-kB p65. Taken together, these data demonstrate for the first time a mechanism for the inactivation of Nrf2 in CF epithelial cells, that Nrf2-regulated proteins markedly affects \( \text{H}_2\text{O}_2 \) levels (12), we measured Nrf2 activity by using 2D SDS-PAGE to examine the expression of seven proteins under its transcriptional control (26). We also measured Nrf2 activity by using 2D SDS-PAGE to examine the expression of seven proteins under its transcriptional control, as previously described (7). The densities of 2D gel bands for peroxiredoxin (PRDX) 1 and 6, thioredoxin (TRX) 1, GST1, catalase (CAT), and NAD(P)H dehydrogenase quinone (NQO) 1 were measured by a BioRad GS-800 densitometer using the 2D gel analysis software PD Quest. PRDX proteins and catalase play a major role in regulating \( \text{H}_2\text{O}_2 \) levels in cells.

### MATERIALS AND METHODS

**Materials.** Cell culture media were purchased from Invitrogen (Carlsbad, CA). Reagents for reverse-phase liquid chromatography and mass spectrometric analysis were supplied by Thermo Fisher Scientific (Waltham, MA). Lipofectamine was purchased from Invitrogen. Luciferase assay reagent was purchased from Promega (Madison, WI). Nrf1 and Nrf2 promoter-driven reporter plasmids were purchased from Panomics (Fremont, CA). NF-kB promoter-driven luciferase reporter plasmid was purchased from Clontech Laboratories (Mountain View, CA). Intracellular \( \text{H}_2\text{O}_2 \) was measured using Amplex Red purchased from Invitrogen. Western blotting for Nrf2 was conducted with an antibody generated in rabbits against a His6-S-tagged full-length Nrf2 protein expressed and purified from bacteria. Alternatively, we used the following antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, CA): for Nrf2, the H300 antibody; for CBP, \( \beta \)-actin, and Lamin B, we used the sc-1211, AC-15, and C-20 antibodies, respectively; for NF-kB p65, we used the SC-7151 antibody; and for secondary antibodies, we used ones conjugated to horseradish peroxidase (HRP). Rp-cAMPS was purchased from BioLog (Hayward, CA).

**Cell culture.** We tested three types of cultured epithelia, two immortalized cell line pairs and one primary cell model. For the immortalized cells, we studied the 16HBEo short sense (S) and antisense (AS) or the 9HTEo pCEP and pCEP-R matched cell pairs. Cell pair identity was confirmed by Western blotting for CFTR for the 9HTEo or the R-domain for the 16HBEo cells. The 16HBEo matched cell line pair was derived from human bronchial epithelia (HBE) and was stably transfected with S or AS of the CFTR nucleotides 1–131. The 9HTEo cell line pair was derived from human tracheal epithelia (HTE) and was stably transfected with pCEP (empty vector) or pCEP-R (pCEP vector encoding the regulatory domain of CFTR). Both cell lines were cultured as previously described (17). For studies in primary cells, we used CF or non-CF HBE grown at an air-liquid interface on semipermeable filters (gift from Chantest, Cleveland, OH). Once plated, the cells were cultured in media containing Ultraser G (Biosepra, Paris, France). The cells were allowed to become differentiated and form tight junctions, as assessed by resistance measurements, and then studied.

**Cell treatments.** Cell lines and primary cells in culture were treated with Rp-cAMPS, TNF-\( \alpha \), or IL-1\( \beta \). For all treatments, media was replaced every 24 h with fresh media containing fresh reagents. For experiments with primary epithelia, reagents were added to both the apical and basolateral compartments and replaced daily. For experiments testing the effect of the inhibition of cAMP-mediated signaling, cells were treated with Rp-cAMPS as previously described (26). Briefly, Rp-cAMPS was added at a final concentration of 50 \( \mu \text{M} \) to media for 72 h. For experiments on the effect of inflammatory stimulation, 10 ng/ml each of TNF-\( \alpha \) and IL-1\( \beta \) were added to the media overnight, and the cells were processed for assays the following day. No significant increase in cell death was observed in any treated vs. untreated cells at 72 h, as assessed by Trypan blue staining.

**Analysis of Nrf2 activity.** We tested Nrf2 activity by two methods: reporter gene expression driven by an ARE promoter and proteomic analysis of a subset of antioxidant proteins whose expression is regulated by Nrf2. For reporter gene expression studies, cell line models were transfected cell line models with a firefly luciferase expression plasmid (pNrf2-fluc) under the control of the Nrf2 promoter for glutathione-S-transferase (GST) 1. To control for transfection efficiency between cell lines, we used a Renilla luciferase expression plasmid under the control of the cytomegavirus (CMV) promoter (pCMV-rluc). Cells were grown to 80% confluence in 24-well plates and transfected with DNA complexed with lipofectamine, as previously described (10). A sample (1 \( \mu \)g) of total DNA (0.9 \( \mu \)g pNrf2-luc + 0.1 \( \mu \)g pCMV-rluc) was used for the transfection of each sample well. Three hours following transfection, cells were washed and media was replaced. One day following transfection, cells were homogenized and the homogenates centrifuged at 14,000 \( \times \)g. Supernatants were collected and examined for firefly and Renilla luciferase activity by dual luciferase assay. Firefly luciferase data (Nrf1 or Nrf2 promoter activity) were normalized to Renilla luciferase values.

We also measured Nrf2 activity by using 2D SDS-PAGE to examine the expression of seven proteins under its transcriptional control, as previously described (7). The densities of 2D gel bands for peroxiredoxin (PRDX) 1 and 6, thioredoxin (TRX) 1, GST1, catalase (CAT), and NAD(P)H dehydrogenase quinone (NQO) 1 were measured by a BioRad GS-800 densitometer using the 2D gel analysis software PD Quest. PRDX proteins and catalase play a major role in regulating \( \text{H}_2\text{O}_2 \) levels in cells.

**Measurement of intracellular \( \text{H}_2\text{O}_2 \).** Because the expression of Nrf2-regulated proteins markedly affects \( \text{H}_2\text{O}_2 \) levels (12), we measured \( \text{H}_2\text{O}_2 \) in a number of experiments. This outcome is also an important link to changes in inflammation, as \( \text{H}_2\text{O}_2 \) is a potent modulator of inflammatory signaling cascades (44). We used the...
Amplex Red assay to assess levels of intracellular H$_2$O$_2$, as previously described (7). The assay has a limit of detection of 50 nM H$_2$O$_2$. Briefly, following treatment with Rp-cAMPS, TNF-α, and/or IL-1β for specified times, cells were rinsed, rapidly lysed, and analyzed or frozen immediately. For analysis, an aliquot of 25 μl of cell lysate was mixed with 25 μl of working solution containing 100 μM Amplex Red reagent and 0.2 U/ml HRP and incubated for 30 min at room temperature. Standards and samples are then analyzed for fluorescence levels at an emission wavelength of 590 nm (excitation, 544 nm). Levels of intracellular H$_2$O$_2$ were normalized to protein concentration.

Subcellular protein fractionation. We used fractionation to examine the levels of Nrf2 in different compartments of cell line and primary cell models. We purified cytoplasmic, membrane, nuclear, and cytoskeletal fractions from cultured cells (cell lines and primary cells) using a subcellular protein fractionation kit (Pierce kit no. 78840; Thermo Fisher Scientific). Cells were harvested using 0.25% trypsin-EDTA, centrifuged at 500 g for 5 min, resuspended in ice-cold PBS, and then centrifuged at 500 g. Each fractionation was carried out on a 10-μl volume of packed cells. Cells were resuspended in 100 μl of ice-cold cytoplasmic extraction buffer and incubated at 4°C for 10 min with gentle mixing. Following centrifugation at 500 g for 5 min, the supernatant (cytoplasmic fraction) was collected. Nuclear extraction buffer (100 μl) was then added to the pellet and the sample vortexed vigorously, incubated at 4°C for 10 min, and centrifuged at 3,000 g for 5 min. The supernatant (membrane fraction) was collected. Nuclear extraction buffer (100 μl) was then added to the pellet and the sample vortexed vigorously, incubated at 4°C for 30 min, centrifuged at 5,000 g, and the supernatant (nuclear fraction) collected. All extracts were stored at 4°C overnight and examined by Western blot the next day.

Animal experiments. Studies conducted in animals were reviewed and approved by the Case Western Reserve University Institutional Animal Care and Use Committee (IACUC). For studies on the association of CBP with p65 and Nrf2, and/or Nrf2 in the human epithelial 9HTEo cell line pairs and nasal epithelia were excised as previously described (42). Cells or tissues excised from CF cells (7). This deficit in Nrf2-mediated cytoprotection in CF patients was demonstrated that cholesterol accumulation in CF cells was reversible upon treatment with the cAMP-binding competitor Rp-cAMPS, suggesting that an aspect(s) of cAMP-mediated signaling contributes to CF cellular phenotypes (26). We have also shown that CF cells exhibit a significant decrease in Nrf2 activity, and this has a profound impact on redox regulation in CF cells (7). This deficit in Nrf2-mediated cytoprotection in CF epithelia contributes to inflammation, insufficient expression of cAMP signaling modulates Nrf2 activity. Previously, we demonstrated that cholesterol accumulation in CF cells was reversible upon treatment with the cAMP-binding competitor Rp-cAMPS, suggesting that an aspect(s) of cAMP-mediated signaling contributes to CF cellular phenotypes (26). We have also shown that CF cells exhibit a significant decrease in Nrf2 activity, and this has a profound impact on redox regulation in CF cells (7). This deficit in Nrf2-mediated cytoprotection in CF epithelia contributes to inflammation, insufficient expression of...
Nrf2 target genes, and a diminished antioxidant response (7). The mechanism of Nrf2 dysregulation in CF cells is not clear, but it is known that, under normal conditions, cAMP-mediated signaling can modulate Nrf2 activity (13, 22). Therefore, we investigated the influence of cAMP-mediated signaling on Nrf2 activation in CF model cells. When assessed by Nrf2 promoter-driven luciferase expression, 9HTEo pCEPR (CF) and 16HBEo AS (CF) cell pairs exhibit significantly lower levels of Nrf2 activity (by 52.4% and 34.8%, respectively) than non-CF matched controls, in the absence of treatment (Fig. 1A). Rp-cAMPS signifi-

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**Fig. 1. Rp-cAMPS corrects nuclear-factor-E2-related factor 2 (Nrf2) activity in cystic fibrosis (CF) airway epithelia toward non-CF levels.** To measure Nrf2 transcriptional activity, CF cell lines and non-CF matched controls were transfected with a firefly luciferase expression cassette under the control of a Nrf2 promoter and a Renilla luciferase cassette under the control of the cytomegalovirus (CMV) promoter (transfection control). To examine the consequence of Nrf2 activity, the whole cell expression levels of proteins under the control of Nrf2 were analyzed by 2D gel electrophoresis. Both Nrf2 promoter activity and Nrf2-regulated protein expression were analyzed in the absence or presence of 50 μM Rp-cAMPS (Rp-cA) for 72 h. A: Nrf2 promoter activity normalized to Renilla control in cell line models. B: Nrf1 promoter activity normalized to Renilla control in CF cell lines. C: whole cell β-actin and Nrf2-regulated protein expression in cell line models (n = 12). D: whole cell β-actin and Nrf2-regulated protein expression in CF primary cells (n = 3). *Significant difference (P < 0.05) from non-CF control. **Significant difference (P < 0.05) from untreated CF matched cells. Error bars represent SE. AS, antisense; TRX1, thioredoxin; GST1, glutathione-S-transferase; NQO1, NAD(P)H dehydrogenase quinone; CAT, catalase; PRDX, peroxiredoxin; HBE, human bronchial epithelium; HTE, human tracheal epithelium.
significantly reversed this defect in CF cell pairs, producing an increase in Nrf2 activity of 83.7–104% compared with untreated CF cells (Fig. 1A). Nevertheless, Rp-cAMPS failed to completely return Nrf2 activity to normal levels (Fig. 1A). The Rp-cAMPS effect was specific to Nrf2, as no change in Nrf1 activity was detected by luciferase reporter assay in CF or non-CF matched cell pairs (Fig. 1B).

To further examine Nrf2 function, we used 2D gel SDS-PAGE and proteomic analyses to determine the levels of proteins under the control of Nrf2 transcriptional activity. We first examined the 9HTeo− cell line pair. Consistent with our previous findings (7), we found a significant decrease in the expression of a number of Nrf2 target gene products, including TRX1, GST1, NQO1, and PRDX1, 3, and 6 (Fig. 1C). Decreases in these proteins at a whole cell level ranged from 2.46–6.32-fold in CF vs. non-CF cells (Fig. 1C). Treatment with Rp-cAMPS partially corrected the decrease in TRX1, GST1, NQO1, CAT, and PRDX1 and 6 (Fig. 1C). Rp-cAMPS had no impact on β-actin levels (Fig. 1C), which is not regulated by Nrf2. We observed a similar effect of Rp-cAMPS in CF primary epithelial cells grown on filters at an air-liquid interface (Fig. 1D). These data are consistent with the activation of Nrf2 by Rp-cAMPS observed in the luciferase reporter studies and demonstrate an Nrf2-mediated functional impact of modulating cAMP-mediated signaling in CF cells.

Rp-cAMPS reduces peroxide production in CF cell lines and primary cells. Our reporter gene and proteomic analyses indicated that Rp-cAMPS activates Nrf2 and influences the expression of a number of Nrf2 target gene products that can modulate the steady-state levels of H2O2 in the cell. Previously, we showed that H2O2 is a potent proinflammatory mediator (7). This proinflammatory effect is attributed to a number of functions of H2O2 in cells, including facilitating TNF-α (8, 20) and IL-1β (19) receptor signaling cascades, and the direct modification and activation of NF-κB (25, 36). Given the importance of inflammatory signaling in CF pathology, and its sensitivity to H2O2 levels, which are influenced by Nrf2 activity, we measured the impact of Rp-cAMPS on steady-state levels of H2O2 in our cell-culture models. We found that, in both CF cell lines and primary CF epithila that have been stimulated with TNF-α and IL-1β, treatment with Rp-cAMPS significantly decreased steady-state H2O2 (Fig. 2). Following treatment with Rp-cAMPS, H2O2 levels in stimulated 9HTeo− pCEPR cells decreased by 53.6%, whereas levels in stimulated 16HBeo− AS cells were reduced by 32.9%, compared with untreated controls (Fig. 2A). Notably, Rp-cAMPS had little influence on H2O2 production in non-CF cell pairs. These observations extended to our primary CF cell model (Fig. 2B), in which Rp-cAMPS decreased steady-state intracellular H2O2 levels by 23–55% in three different donor vs. untreated same-donor controls. Taken together, our findings are consistent with partial and significant activation of Nrf2 by Rp-cAMPS. These studies also reveal that treatment with Rp-cAMPS elicits a CF-cell-specific inflammation-related functional outcome of modulating cAMP-mediated signaling with no apparent effect on normal control cells.

Rp-cAMPS increases nuclear levels of Nrf2 independently of Keap1 expression. To further examine the mechanism by which Rp-cAMPS rescues Nrf2 function in CF cells, we examined Nrf2 localization in response to treatment. Levels of Nrf2 protein in the AS (CF) cell pair are slightly diminished in the cytoplasmic fraction compared with S (non-CF) controls (Fig. 3A). A significant decrease of Nrf2 accumulation in the nuclear fraction was observed in CF cells (Fig. 3A). Similar results were observed in the pCEPR (CF)/pCEP (non-CF) cell pair (Fig. 3B), as well as in primary CF epithelia compared with non-CF controls (Fig. 3C). Treatment with Rp-cAMPS only impacted cytoplasmic levels of Nrf2 in the S (non-CF)/AS (CF) cell line pair (Fig. 3A) but did not have a significant impact on cytoplasmic levels in the pCEPR (CF)/pCEP (non-CF) cell line pair or primary CF cells (Fig. 3, B and C, respectively). A robust impact of Rp-cAMPS on nuclear levels of Nrf2 in all CF cell models was observed (Fig. 3). These findings are consistent with the decrease in Nrf2 function in CF vs. non-CF cells observed in our Nrf2 promoter analyses and the examinations of Nrf2-regulated protein expression. Further studies revealed that the levels of Keap1 in CF cells were not influenced by Rp-cAMPS (Fig. 3D). This finding indicates that a decreased level of Keap1 is not the chief effect by which Rp-cAMPS activates Nrf2 in CF cells.

cAMP-mediated signaling influences NF-κB activation and cytokine production in CF-model cells. The above data demonstrate that Rp-cAMPS treatment increases Nrf2 function in CF cells by increasing nuclear translocation and/or retention of...
the transcription factor independent of Keap1 levels. Another candidate cofactor related to cAMP-mediated signaling that has been associated with Nrf2 nuclear translocation is CBP (40). Liu et al. (23) reported that the p65 subunit of NF-κB competes with Nrf2 for CBP binding. If Rp-cAMPS influences Nrf2 regulation through CBP, this would predict that Rp-cAMPS should reduce NF-κB activation and subsequent cytokine production in a CF-specific manner. To assess whether the cAMP pathway impacts CF inflammatory signaling, we examined the effect of Rp-cAMPS on NF-κB activation, IL-6...
production, and IL-8 production all in response to stimulation by IL-1β and TNF-α. NF-κB activity, as measured by luciferase reporter assay, was significantly diminished by Rp-cAMPS in 9HTEo-pCEP (CF) cells vs. non-CF controls (Fig. 4A). These findings translated into significant differences at the level of cytokine production. Inflammatory stimulation of the 9HTEo- cell pair resulted in increases in secreted IL-8 and IL-6 levels. Rp-cAMPS had little influence on secreted IL-8 or IL-6 in non-CF control cells but significantly reduced levels in CF-model cells (Fig. 4, B and C). A similar result was observed for primary airway epithelial cells, where Rp-cAMPS treatment significantly decreased IL-8 production by 32.7% in primary CF bronchial epithelia vs. a non-significant decrease in non-CF cells (Fig. 4D). These data indicate that cAMP-related signaling plays a larger role in modulating NF-κB mediated inflammation in CF vs. non-CF epithelial cells.

Rp-cAMPS inhibition of cAMP-mediated signaling modulates CBP interaction with NF-κB and Nrf2. The above studies demonstrate that Rp-cAMPS treatment significantly reduces NF-κB activity and increases Nrf2 activity in CF cells, correcting both respective functions to levels approaching those in non-CF levels. These findings led us to hypothesize that Rp-cAMPS influences CF-related regulation of Nrf2 and NF-κB through a common factor, namely, CBP. We tested this hypothesis using coimmunoprecipitation assays and found that CBP binding to NF-κB p65 is not significantly different in CF cell models vs. non-CF controls (Fig. 5A) and is evident in excised nasal epithelia from F508 mice (Fig. 5B). Conversely, association of CBP with Nrf2 was dramatically diminished in CF cell models (Fig. 6A) and essentially undetectable in excised nasal epithelia from F508 mice (Fig. 6B). Because CBP acts to transactivate both NF-κB and Nrf2, our data are consistent with an increase in NF-κB activity and a concomitant decrease in Nrf2 activity. Treatment with Rp-cAMPS reversed CBP association patterns with NF-κB and Nrf2 in both CF cell models and nasal epithelial cells from CF mice (Figs. 5 and 6, respectively). These data are consistent with the activation of Nrf2 and the inhibition of NF-κB that we observe following treatment with Rp-cAMPS.

**DISCUSSION**

Given the critical role of inflammation in CF disease progression, the current study was designed to identify the molecular pathways that modulate inflammatory signaling in CF epithelial cells. We hypothesized that dysfunction of CFTR
induces a feedback elevation of cAMP-mediated signaling in CF and results in the aberrant regulation of NF-κB and Nrf2, two transcription factors that play central roles in inflammatory signaling. A number of studies have demonstrated that the activities of NF-κB and Nrf2 are inversely linked (23) and that the interplay between these activities controls the severity of the inflammatory response (21, 31). In addition, disruption of Nrf2 and a related increase in NF-κB activity have been associated with increased inflammation in a number of airway diseases with CF-like pathologies, including asthma (38) and chronic obstructive pulmonary disease (35).

Previously, we reported that Nrf2 activity is markedly reduced in CF cell lines and primary cells, as well as in lungs and excised nasal epithelia from CF mice (7). In that work, we reported a decrease in Nrf2 activity of 50–80% vs. non-CF controls. When examined the levels of Nrf2, we found that whole cell levels of Nrf2 were only slightly decreased, whereas levels of Nrf2 in the nucleus were markedly diminished, and this contributed to increased inflammatory responses (7). These observations are consistent with our present studies in primary CF cells grown at an air-liquid interface. Therefore, based on our studies, it is presently our hypothesis that the whole cell levels of Nrf2 in CF cells are not significantly relevant to Nrf2 activity in CF cells, but rather the levels of Nrf2 in the nucleus are the chief contributor to the alteration in activity. Furthermore, in this report, we present in vitro and in vivo evidence that altered interaction with CBP is a primary underlying mechanism for a pathological imbalance in the levels of NF-κB and Nrf2 in the nucleus and consequently significantly favors a pro- rather than anti-inflammatory phenotype. Treatment with a competitor of cAMP can correct abnormalities in NF-κB and Nrf2 activities and interaction with the shared transcriptional coactivator, CBP. This result is consistent with our previous findings that cAMP-mediated signaling is altered in CF cells (26).

Our findings in cultured cell lines and corroborating data from a CF mouse model and human primary CF epithelia indicate that cAMP-mediated signaling in CF cells specifically causes an increase of NF-κB activity coupled to a concomitant decrease in Nrf2 function, contributing to the proinflammatory state in CF. Although additional mechanisms likely contribute to Nrf2 dysregulation, diminished antioxidant capacity, and related excessive inflammatory signaling in CF epithelia, inhibition of cAMP-mediated signaling significantly corrects these abnormalities. Notably, this mechanism links a response to CFTR dysfunction (e.g., altered cAMP-mediated signaling) to the clinically relevant manifestations of excessive inflammation (3, 11, 29) and diminished antioxidant and anti-inflammatory signaling present in CF (6, 7, 39, 41).

Our data indicate that nuclear levels of Nrf2 are decreased in CF. One possible mechanism for this deficiency is that a decreased interaction with CBP decreases nuclear dwell time and
DNA binding of the transcription factor (13, 40). Alternatively, nuclear Nrf2 accumulation may be suppressed by increased levels of Keap1. We do not currently favor this notion, as we did not detect a change in Keap1 levels in CF cells relative to non-CF cells. Further evidence against excessive Keap1-mediated Nrf2 suppression in CF epithelia derives from our previous work showing that CF cells have elevated intracellular levels of H2O2 (7), an environment expected to reduce Keap1-Nrf2 binding (45).

Interestingly, the results in the 9HTEo/H11002 cell line pair model, which recapitulates the phenotypic condition present in epithelia of patients with CF, indicate that feedback cAMP-mediated signaling ultimately causes the dysregulation of NF-κB and Nrf2 in response to a lack of CFTR function. These data are consistent with our previous studies showing that inhibition of CFTR with CFTRinh-172 was sufficient to inhibit Nrf2 activity and thereby increase oxidant-mediated elevations in cytokine levels (7). Recently, Kelly and colleagues (14) concluded that increases in oxidants and inflammatory signaling in CF cells are independent of CFTR channel function loss, as CFTRinh-172 elicited these changes in cells that lack detectable levels of CFTR. However, these studies did show elevated levels of intracellular oxidants in the absence of CFTRinh-172 in CF vs. non-CF controls. Therefore, whereas CFTRinh-172 may have off-target effects, these studies do not preclude an effect from lack of CFTR function in cells that do express the channel.

In CF, the balance between CBP binding to NF-κB and Nrf2 is continually disrupted in a CAMP-dependent manner. Although persistent CAMP-mediated signaling may be present in CF cells as a response to inactive CFTR, the measurement of cAMP levels in CF cells has been very limited, mainly due to methodological difficulties. A single study has reported that levels of cAMP in CF nasal and tracheal epithelia, while trending higher, are not significantly different from non-CF controls (43). Levels of cAMP were not examined following inflammatory stimulation, the condition under which we observe the most significant inhibition of NF-κB by Rp-cAMPS. Noticeably, our findings on the effects of Rp-cAMPS in CF cells are consistent with a decrease in CBP-NF-κB p65 complex formation and a concomitant increase in CBP availability for binding to Nrf2. The consequence of this switch is a decrease in inflammatory signaling and an increase in antioxidant/anti-inflammatory activity. Thus the restoration of CBP distribution between Nrf2 and NF-κB represents a potential new avenue of therapeutic intervention.

Our test-compound Rp-cAMPS, although effective, is not a likely therapeutic agent because of the broad importance of the CAMP pathway in many physiological systems. We have noted in many of our treated animals slowed wound healing and some wound dehiscence, likely attributable to the Rp-cAMPS downregulation of inflammation and inhibition of granulation tissue formation. Although this is not entirely surprising, it also suggests that, as a therapeutic agent, Rp-cAMPS would likely have a narrow therapeutic window between the desired outcome of decreased airway inflammation and of potential adverse effects. These unwanted effects could potentially be limited by alternative modes of delivery (i.e., nebulization), but further study of Rp-cAMPS and its mechanism of action will be necessary. Nevertheless, the efficacy of Rp-cAMPS points...
to a potential impact of cAMP feedback-signaling in CF and may point to other sites of intervention that are more feasible, including strategies to mitigate the interaction between CBP with NF-κB p65. Alternatively, approaches that enhance the activity of Nrf2, especially by promoting its interaction with CBP may be desirable for CF therapy. The clear implication of this study is that therapeutically targeting CBP interactions should be considered.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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