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# Impaired TLR4 and HIF expression in cystic fibrosis bronchial epithelial cells downregulates hemeoxygenase-1 and alters iron homeostasis in vitro

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**Chillappagari S, Venkatesan S, Garapati V, Mahavadi P, Munder A, Seubert A, Sarode G, Guenther A, Schmeck BT, Tümmler B, Henke MO.** Impaired TLR4 and HIF expression in cystic fibrosis bronchial epithelial cells downregulates hemeoxygenase-1 and alters iron homeostasis in vitro. *Am J Physiol Lung Cell Mol Physiol* 307: L791–L799, 2014. First published September 19, 2014; doi:10.1152/ajplung.00167.2014.—Hemeoxygenase-1 (HO-1), an inducible heat shock protein, is upregulated in response to multiple cellular insults via oxidative stress, lipopolysaccharides (LPS), and hypoxia. In this study, we investigated in vitro the role of Toll-like receptor 4 (TLR4), hypoxia-inducible factor 1α (HIF-1α), and iron on HO-1 expression in cystic fibrosis (CF). Immunohistochemical analysis of TLR4, HO-1, ferritin, and HIF-1α were performed on lung sections of CFTR−/− and wild-type mice. CFBE41o- and 16HBE14o- cell lines were employed for in vitro analysis via immunoblotting, immunofluorescence, real-time PCR, luciferase reporter gene analysis, and iron quantification. We observed a reduced TLR4, HIF-1α, HO-1, and ferritin in CFBE41o- cell line and CF mice. Knockdown studies using TLR4-siRNA in 16HBE14o- revealed significant decrease of HO-1, confirming the role of TLR4 in HO-1 downregulation. Inhibition of HO-1 using tin protoporphyrin in 16HBE14o- cells resulted in increased iron levels, suggesting a probable role of HO-1 in iron accumulation. Additionally, sequestration of excess iron using iron chelators resulted in increased hypoxia response element response in CFBE41o- and 16HBE14o-, implicating a role of iron in HIF-1α stabilization and HO-1. To conclude, our in vitro results demonstrate that multiple regulatory factors, such as impaired TLR4 surface expression, increased intracellular iron, and decreased HIF-1α, downregulate HO-1 expression in CFBE41o- cells.

iron; Toll-like receptor 4; hypoxia-inducible factor 1α; cystic fibrosis

CYSTIC FIBROSIS (CF) is an autosomal recessive genetic disorder caused by a mutation in the gene found at the q31.2 locus of chromosome 7, which encodes the CF transmembrane conductance regulator (CFTR) protein. CF is a multi-organ disease, but lungs are the major site of morbidity and mortality (2, 6). Normal individuals have two functional copies of the *CFTR* gene, but one functional copy is sufficient to prevent the disease (38). The most common CF mutation (60–70%) is the in-frame deletion of phenylalanine (F) at 508 position (ΔF508) of the CFTR protein. This yields an abnormally folded CFTR protein that fails to be targeted to the plasma membrane (4). This subsequently results in impaired epithelial chloride ($Cl^-$) ion channeling, sodium ($Na^{2+}$)-dependent water transport, and decreased clearance of mucus secretion (14, 34). These thick mucus plaques act as a nidus for persistent bacterial infection, resulting in increased airway inflammation and oxidative stress (1).

Hemeoxygenase-1 (HO-1) is a stress-inducible protein with potential anti-inflammatory and antioxidant properties (31). HO-1 catalyzes the oxidative degradation of heme into carbon monoxide (CO), iron ($Fe^{2+}$), and biliverdin IXα, which in turn is converted to bilirubin IXα, and these byproducts are shown to exhibit anti-inflammatory and antioxidant properties (5). HO-1 is induced in response to various stressful stimuli, such as lipopolysaccharide (LPS), heme, and UV radiation (7). LPS induces HO-1 via Toll-like receptor (TLR4) in multiple cell types, implicating its role in curbing inflammation (39). Mice with targeted deletion of HO-1 resulted in a chronic state of inflammation. Apart from inflammation, HO-1 plays a pivotal role in regulating cellular iron (33). HO-1−/− mice displayed increased accumulation of iron in the liver, and overexpression of HO-1 reduced the intracellular iron by increasing iron efflux in cultured fibroblasts (11). HO-1-deficient human patients demonstrated multiple abnormalities, such as anemia, abnormal iron metabolism with increased iron deposition, and growth retardation (41). Increased iron has also been reported in the explanted lungs and lavages of patients with CF (13).

It is well documented that patients with CF demonstrate deteriorated lung function attributable to a vicious infection-inflammation cycle and thick mucus accumulation on the airway epithelial surface (26). This mucus accumulation is also driven by activation of HIF-1α, and accumulation of mucins in CF airways during pulmonary exacerbation might lead to impaired gaseous exchange (17, 43). The activity of HIF-1α is dependent on iron and oxygen (9). In addition, increased HO-1 expression was observed when rats were exposed to hypoxia

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(7%) (28), indicating that HIF-1α transcriptionally activates HO-1 under hypoxia. The current study aims to investigate the possible role of TLR4, iron, and HIF-1α on the expression of HO-1 in an in vitro model of CF (CFBE41o-) and to elucidate a potential link between them in CF.

## MATERIALS AND METHODS

*Cell culture.* Human bronchial epithelial cell line with defective ΔF508 CFTR (CFBE41o-) and normal human bronchial epithelial cell lines (16HBE14o-) were obtained from Dr. Dieter Gruenert. Cells were cultured according to protocols described before (20). siRNA knockdown studies were performed on cells cultured in six-well tissue culture plates (Greiner Bio-One) as described earlier (22), except that the transfection reagent was replaced with Dharmafect (Dharmacon). Cells were either treated with LPS (2, 5, and 10 μg/ml) from *Pseudomonas aeruginosa* (*P. aeruginosa*) (Sigma-Aldrich), iron III citrate (50 μM; Sigma-Aldrich), or iron chelator (50, 100, 200 μM) (2,2′-dipyridyl, 2,2′-DP) (Fluka Analytical). Controls were treated with PBS only. Hypoxic treatments were performed by growing the cells in hypoxia incubator maintained at 0.1% oxygen. All transfections and treatments were performed in triplicate.

*RNA isolation and RT-PCR.* mRNA expression of HO-1 was analyzed by quantitative real-time RT-PCR. Total RNA from cells was isolated using the Trizol method and phase lock separation tubes. The concentration and quality of RNA was determined using NanoDrop 2000c photometer (PeqLab). cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems) according to manufacturer's protocol. Generated cDNA was used as a template for amplification in an qRT-PCR (ViiA 7 Real-Time PCR System, Applied Biosystems), using SYBR Select Master Mix (Applied Biosystems) according to manufacturer's protocol. HO-1-specific primers were generated using genetool software from respective human mRNA coding sequences obtained from NCBI (National Center for Biotechnology Information) database and were synthesized by Eurofins, Germany; sequences are listed in Table 1. The PCR was programmed for initial denaturation (95°C for 10 min) followed by 40 cycles of denaturation (95°C for 15 s), annealing at 60°C for 1 min. Melting curve analysis was performed in between 50° and 95°C, which reads the plate for every 0.1°C after holding the temperature for 5 s. $\Delta\Delta C_T$ analysis was used to calculate expression compared with housekeeping control (β-actin) and was normalized to the level of 16HBE14o- cells.

*Western blotting and immunohistochemistry.* Cells were lysed using lysis buffer containing 50 mM Tris, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% Na-deoxycholate, and 1 mM PMSF. The lysates were disrupted by sonication, and total protein concentrations were measured using the Pierce BCA protein estimation kit according to manufacturer's protocol. Cytosolic protein extracts (25 μg total protein per lane) were separated by SDS-PAGE before being blotted onto PVDF membranes. Membranes were blocked with buffer containing 50 mM NaCl and 50 mM Tris·HCl (pH 7.5) containing 5% (wt/vol) milk. Proteins TLR4, ferritin (Santa Cruz Biotechnology), HO-1, and β-actin (Sigma) were detected using specific polyclonal primary antibodies obtained from rabbit and horseradish peroxidase conjugated anti-rabbit IgG (Dako) as a secondary antibody. Lungs of CFTR−/− and the wild-type mice were fixed in 4% paraformaldehyde and embedded in paraffin, and serial sections (3-μm thickness) were performed. AP-fast red kit or horseradish peroxidase-diaminobenzidine kit (Zytochem Systems) were used, and all sections were scanned as described before (1, 2) and according to the manufacturer's instructions for immunohistochemical localization of the following proteins: TLR4 and HO-1 (both Enzo Life Sciences), HIF-1α (LS-Bioscience), and ferritin (Santa Cruz Biotechnology). Antigen retrieval was performed by microwaving the sections in 10 mM sodium citrate buffer, pH 6.0. Hemalaun was used as a counterstain.

Table 1. *Primers (5′-3′) used for mRNA quantification*

| Gene | Primer Sequence (5′-3′) |
|---|---|
| hHO1-FP | 5′-TTCTTCACCTTCCCCAACATTG-3′ |
| hHO1-RP | 5′-CAGCTCCTGCAACTCCTCAAA-3′ |
| hβ-actin-FP | 5′-CATGTACGTTGCTATCCAGGC-3′ |
| hβ-actin-RP | 5′-CTCCTTAATGTCACGCACGAT-3′ |

*Immunofluorescence, transfection of plasmids, and luciferase assay.* Cells were grown to 80–90% confluency, treated with LPS (2 μg/ml) for 4 h, and washed twice with PBS (Gibco), followed by immunofluorescence. Cells were grown and treated in BD falcon culture chamber slides and fixed in 4% paraformaldehyde (Sigma-Aldrich) for 10–15 min at room temperature. Permeabilization was done with 0.1% saponin (Sigma-Aldrich) for 10–15 min at room temperature, followed by blocking with 1% BSA for 30 min at room temperature (diluted from 30% BSA from phenylacetic acid). Without being washed, the cells were treated with primary antibodies TLR4 and HO-1 (Enzo Life Sciences) in 1% BSA (1:250) and incubated overnight at 4°C. The next day, secondary antibodies conjugated with appropriate fluorophores 488 or 555 (Alexa Fluor) were diluted in 1% BSA (1:250) and incubated for 1 h at room temperature. Chamber slides were detached from the glass slide, and nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) before the mounting of the coverslip (VECTASHIELD Mounting Medium with DAPI). Immunofluorescence microscopy was performed using an Axiophot Zeiss immunofluorescence microscope at ×100 magnification. CFBE41o- and 16HBE14o- cells were grown until 60–70% confluence and were transfected using transfection reagent TransIT-LT1 (Mirus Bio) with 1 μg of reporter plasmids [pGL3 Basic, pGL3-HO-1, and pGL3-hypoxia response element (HRE)] and 50 ng of internal control (pRL-SV40 from Promega) in different experimental setups. Cells were either harvested after 24 h or treated with either LPS or iron and 2, 2′-Dipyridyl and hypoxia (0.1% oxygen). Luciferase activity was determined using Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Relative light units of firefly luciferase were normalized against *Renilla* luciferase activity.

*CF mice and handling.* In *Cftr*$^{TgH(neoim)Hgu}$ mice, the exon 10 of the *Cftr* gene had been disrupted by the insertion of the vector pMCIneoPolyA. Because those mice produced low levels of Cftr (8) but showed a mixed genetic background (3) from the original *Cftr*$^{TgH(neoim)Hgu}$ mutant mouse, CF strain CF/3-*Cftr*$^{TgH(neoim)Hgu}$ was established at the Institute of Laboratory Animal Science of the Hannover Medical School by brother-sister mating for more than 40 generations. Next, the congenic mouse inbred strain B6.129P2(CF/3)- *Cftr*$^{TgH(neoim)Hgu}$, which is used in this study, was generated by 40 backcross generations using CF/3-*Cftr*$^{TgH(neoim)Hgu}$ as donor strain and C57BL/6J as recipient strain (37). In the following, D2.129P2(CF/3)-*Cftr*$^{TgH(neoim)Hgu}$/B6.129P2(CF/3)-*Cftr*$^{TgH(neoim)Hgu}$ are called Cftr−/− mice, and syngenic C57BL/6J/DBA/2J mice are called B6/D2 and served as controls. Mice of all strains were bred in the Central Animal Laboratory, Hannover Medical School, Hannover, Germany and maintained there in microisolator cages with filter-top lids at 21 ± 2°C, 50 ± 5% humidity, and a 14-h:10-h light/dark cycle. They were supplied with autoclaved, acidulated water and fed ad libitum with autoclaved standard diet. All animal procedures were approved by the local district governments and carried out according to the guidelines of the German law for the protection of animal life. Before the resection of the lungs, mice were anesthetized with an overdose of xylazine/ketamine and killed via bleeding of the abdominal aorta. Lungs were removed aseptically and put in 4% paraformaldehyde.

*Estimation of total iron concentrations in cells.* Total cytoplasmic iron concentrations were measured using inductively coupled plasma mass spectrometry (ICP-MS). CFBE41o- and 16HBE14o- cells were grown in MEM medium until 80–90% confluence. Cells were detached using 1× trypsin (Gibco) and centrifuged at 1,500 revolution/min for 5 min, and the pellets were washed three times with PBS

buffer pH 7.4 (Gibco) supplemented with 1 mM EDTA and twice with MilliQ water. Cells were dried overnight at 85°C, and the total iron concentration was determined by breaking the cells using nitric acid.

*Statistical analysis.* Densitometry of the immunoblots was performed using ImageJ software (http://rsbweb.nih.gov/nih-image/). Significant difference for immunoblots was evaluated by one-way ANOVA and nonparametric Kruskal-Wallis test ($P < 0.05$). Significant difference for ICP-MS was evaluated by unpaired Student's *t*-test with two-tailed distributions ($P < 0.05$). All analyses were performed by means of GraphPad Prism 4 software.

## RESULTS

*Decreased expression of HO-1 in CF.* We quantified the mRNA and protein levels of HO-1 in bronchial epithelial cells with defective ΔF508 mutation in *CFTR* gene (CFBE41o-) and normal human bronchial epithelial cells (16HBE14o-) cells. Under normal conditions (without LPS stimulation), real-time analysis of HO-1 mRNA showed approximately fourfold lower levels in CFBE41o- cells compared with 16HBE14o- cells (Fig. 1*A*). We observed a dose-dependent increase in HO-1 mRNA levels upon stimulation with increasing concentrations of LPS (2, 5, and 10 μg/ml) for 8 h; however, the overall expression of HO-1 in CFBE41o- cells still remained threefold lower at 10 μg/ml LPS compared with 16HBE14o- cells at 10 μg/ml (Fig. 1*A*). Results obtained from immunoblot analysis for HO-1 protein in both cell lines without stimulation also revealed decreased HO-1 in CFBE41o- cells compared with 16HBE14o- cells. Upon stimulation with LPS (2 μg/ml) for different time intervals (2, 4, 6, and 8 h), we observed a gradual decrease in HO-1 protein level in CFBE41o- cells (Fig. 1*B*), which further supports the mRNA data observed at 2 μg/ml for 8 h (Fig. 1*A*), whereas no significant differences in HO-1 expression were observed in 16HBE14o- cells under normal and stimulated conditions (Fig. 1, *B* and *C*). Quantitative analysis of the HO-1 expression using HO-1 luciferase reporter plasmid revealed a fivefold lower activity (without LPS) and a sevenfold lower activity (with LPS) in CFBE41o- cells compared with 16HBE14o- cells. No significant differences in the luciferase activity were observed within CFBE41o- cells upon LPS stimulation (Fig. 1*D*). Furthermore, immunofluorescence analysis of HO-1 without LPS stimulation showed a diffused staining pattern in CFBE41o- cells, whereas condensed staining was observed in 16HBE14o- cells. Upon stimulation with LPS treatment, CFBE41o- cells showed no or little punctate staining in contrast to 16HBE14o-, where significant punctuate localization was observed (Fig. 2*A*). Furthermore, immunohistochemistry analysis of HO-1 performed on lung sections of CFTR−/− mice revealed a drastic decrease in HO-1 staining in the airway epithelial cells compared with the wild-type (Fig. 2*B*). Together, these results confirm decreased HO-1 expression in CFTR-deficient murine and human airway epithelial cells.

*TLR4-dependent downregulation of HO-1 in CF.* Our previous studies on TLR4 in CF and the current literature led us to think that reduced TLR4 surface expression in CF might be

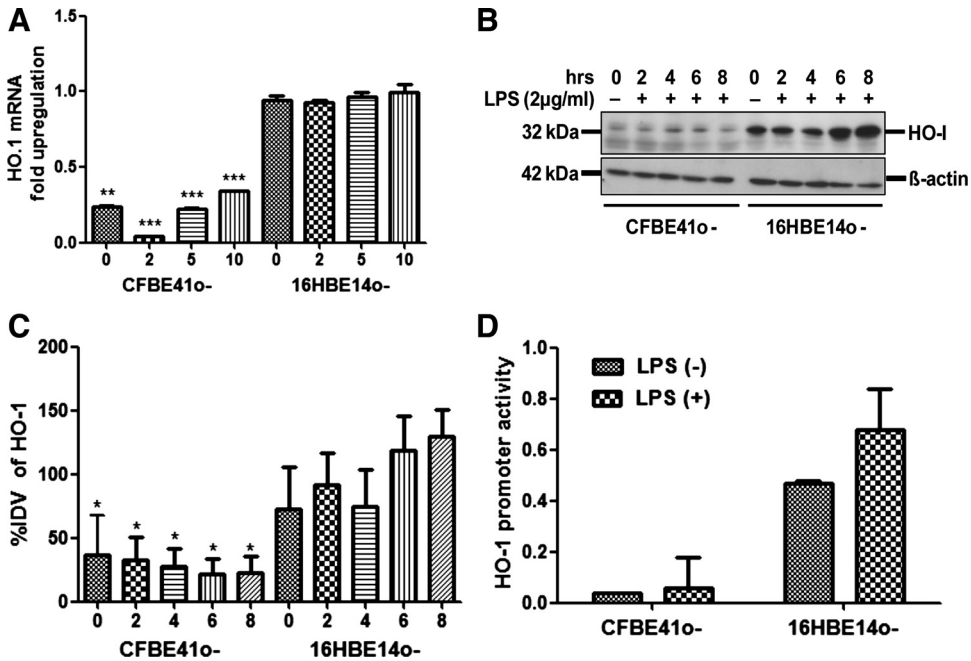


Fig. 1. Hemeoxygenase-1 (HO-1) expression in CFBE41o- and 16HBE14o- cells. *A*: quantification of HO-1 mRNA in CFBE41o- and 16HBE14o- cells upon stimulation with increasing concentrations of LPS (2, 5, and 10 μg/ml) using real-time PCR. HO-1 transcripts of CFBE41o- and 16HBE14o- were normalized with their respective β-actin transcripts (internal control). HO-1/β-actin of 16HBE14o- were compared with the transcripts of HO-1/β-actin from CFBE41o-. Error bars were obtained from 4 independent standard deviations. Data represent means ± SE, and asterisks indicate statistical significance determined by Student's *t*-test (***$P < 0.001$). Significance is relative to respective concentration of LPS for each cell line unless otherwise indicated with bars. *B*: Western blot detection of HO-1 in CFBE41o- and 16HBE14o- cells upon treatment with 2 μg/ml LPS for indicated time intervals. Immunoblotting of β-actin was performed as loading control. *C*: densitometry analysis of HO-1 immunoblotting. HO-1-to-β-actin ratio was calculated and is represented as bar graphs. Error bars were obtained from 6 independent standard deviations. Data represent means ± SE, and asterisks indicate statistical significance determined by Student's *t*-test (*$P < 0.05$). Significance was calculated and is relative to respective time points for each cell line. IDV, intensity density values. *D*: quantification of HO-1 expression in CFBE41o- and 16HBE14o- in the absence or presence of 2 μg/ml LPS overnight using HO-1 luciferase reporter gene expression analysis ($n = 4$). Fold expression is represented after normalization of the expression values with internal control.

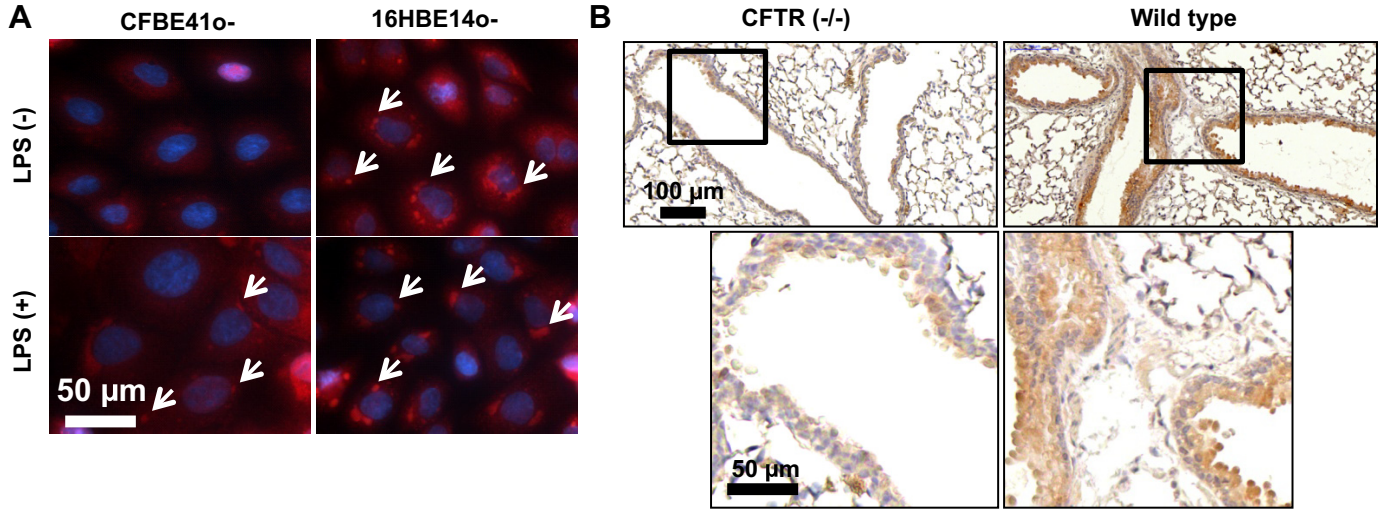


Fig. 2. HO-1 staining in CFBE41o- and 16HBE14o- cells and cystic fibrosis transmembrane conductance regulator (CFTR) (−/−) and wild-type mice lungs. *A*: immunofluorescence analysis of HO-1 (26) in CFBE41o- and 16HBE14o- cells upon LPS treatment (2 μg/ml) for 4 h. Untreated cells served as control. Nuclei are stained with DAPI (blue) ($n = 6$). *B*: immunohistochemistry was performed for HO-1 (brown) on paraffin-embedded lung sections from CFTR −/− ($n = 6$) and wild-type mice ($n = 6$). Nuclei are stained blue.

responsible for downregulated HO-1 expression (11, 20, 22). To support this finding, we performed immunohistochemical analysis of TLR4 in the CFTR−/− and wild-type mice, which revealed no detectable staining for TLR4 in the airway epithelium of CFTR−/− mice, whereas prominent staining for TLR4 in the airway epithelial cells of wild-type mice was observed (Fig. 3*A*). In vitro, immunofluorescence analysis of TLR4 in CFBE41o- cells also showed a diffused staining pattern similar to that observed for HO-1 immunofluorescence in CFBE41o- under unstimulated conditions. Upon stimulation with LPS, we observed a slight localization of TLR4 in CFBE41o- (Fig. 3*B*). In contrast, 16HBE14o- cells displayed a prominent staining for TLR4 similar to that of HO-1 staining in 16HBE14o- without stimulation, whereas enhanced localization of TLR4 in 16HBE14o- cells was seen upon LPS stimulation (Fig. 3*B*). To test the hypothesis that the anomalous TLR4 expression might be responsible for the low HO-1 expression in CFTR-deficient cells, we performed TLR4 knockdown using TLR4-specific siRNA in 16HBE14o- cells. As expected, knockdown of TLR4 resulted in low HO-1 expression (Fig. 3*C*). These results indicate that reduced TLR4 expression in CF might be responsible for downregulation of HO-1.

*HO-1 alters iron homeostasis in CF.* To find a potential link between downregulated HO-1 and accumulation of iron in CF, total intracellular iron content of CFBE41o- and 16HBE14o- cells was quantified using ICP-MS. We observed ~2.5-fold excess iron in CFBE41o- cells compared with 16HBE14o- cells (Fig. 4*A*). To test whether downregulated HO-1 has a direct impact on iron overload, we treated 16HBE14o- cells with 5 μg/ml HO-1 inhibitor (tin protoporphyrin, SnPP) and quantified iron using ICP-MS. We observed a twofold increase in iron content in 16HBE14o- cells treated with SnPP (Fig. 4*B*). This result suggests that downregulated HO-1 might lead to increased iron content. In response to this altered iron homeostasis, we tried to analyze the expression of a major antioxidant and iron-storage protein, ferritin, which is upregulated in response to HO-1 expression (27). We observed a reduced expression of ferritin in CFBE41o- cells, compared with 16HBE14o- cells (Fig. 4, *C* and *D*). Similarly, immunohistochemical analysis revealed no detectable ferritin in the airway epithelium of CFTR−/− mice compared with wild-type mice (Fig. 5). These results provide evidence that increased iron levels in CF might be attributable to downregulated HO-1 and ferritin.

*Iron induced altered hypoxic response in CF.* Previous studies reported by other groups suggest that the stability of HIF-1α is dependent on iron and oxygen (9). To test whether increased iron levels in CF might lead to HIF-1α destabilization and subsequently HO-1, we quantified the hypoxic response in both cell lines using an HRE-luciferase reporter gene assay. Under normal conditions, we observed impaired HRE response in CFBE41o- cells compared with 16HBE14o- cells. In response to hypoxia (0.1% $O_2$), we observed only a twofold increase in promoter activity in CFBE41o- cells in contrast to the eightfold increase observed in 16HBE14o-. Thus 16HBE14o- cells showed a 10-fold higher hypoxic response compared with the CFBE41o- (Fig. 6*A*). We then tested whether altered iron in CFBE41o- alters the hypoxic response. HRE-luciferase reporter plasmid transfected cell lines were grown in an iron-depleted state (2,2′-DP, 50 μM), which resulted in in a 19- and 23-fold increase of promoter activity in CFBE41o- and 16HBE14o- cells, respectively. Repletion with 50 μM iron silenced promoter activity in both cell lines (Fig. 6*A*). No cell death was observed before or after the treatments. To verify whether hypoxia or iron-mediated alteration of HIF-1α might influence HO-1 expression, we tested the expression of HO-1 in CFBE41o- and 16HBE14o- cells under hypoxic conditions (0.1% $O_2$). We observed a significant increase in HO-1 in 16HBE14o- cells, which was further enhanced upon LPS challenge. On the contrary, only a negligible amount of HO-1 was expressed in CFBE41o- under hypoxia (with and without LPS) (Fig. 6*B*). Furthermore, to test the role of iron on HO-1 expression via HIF-1α, we performed immunoblotting and mRNA analysis of HO-1 upon iron chelation. Results obtained from RT-PCR revealed a significant upregulation of HO-1 mRNA in a dose-dependent manner in both the cell lines,

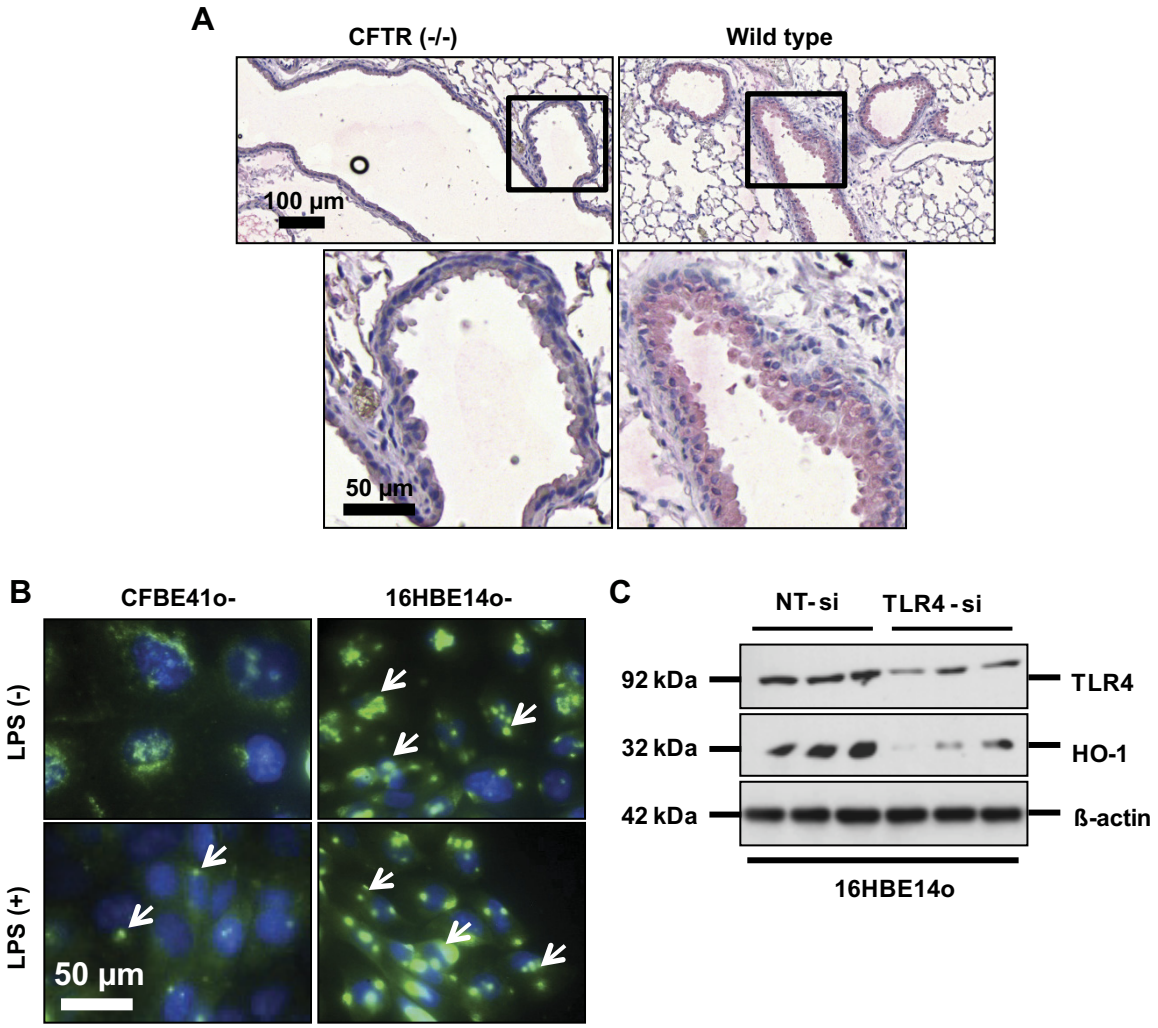


Fig. 3. Toll-like receptor 4 (TLR4) staining in CFBE41o- and 16HBE14o- cells and CFTR (−/−) and wild-type mice lungs. *A*: immunohistochemical analysis of TLR4 (pink) on paraffin lung sections of CFTR (−/−) ($n$ = 6) and wild-type mice ($n$ = 6). Nuclei are stained blue. *B*: TLR4 staining (green) using immunofluorescence in CFBE41o- and 16HBE14o- cell lines upon LPS (2 μg/ml) stimulation for 4 h ($n$ = 6). Untreated cells served as control. Nuclei are stained with DAPI (blue). *C*: Western blot analysis of TLR4 in 16HBE14o- cells treated with 2 μM nontargeting ($NT_{si}$) or TLR4 siRNA ($TLR4_{si}$) for 24 h. Immunoblotting of β-actin was performed as loading control.

and we also observed a slight increase in HO-1 at protein level in CFBE41o- cells, whereas, in 16HBE14o- cells, it remained unaltered (Fig. 6*C*). We also observed a significant increase in upregulation of HO-1 mRNA and protein under hypoxic conditions in both cell lines compared with normal conditions (Fig. 6, *D* and *E*). These results suggest that hypoxia significantly contributes to upregulation of HO-1. Additionally, we performed the immunohistochemical analysis of HIF-1α in CFTR−/− and wild-type mice. We observed very low HIF-1α in the airway epithelium of CFTR−/− mice compared with high HIF-1α levels in wild-type mice (Fig. 7). These findings coincide with our immunoblot and HRE reporter assays and suggest a pivotal role of iron in the altered HIF-1α and HO-1 in CFBE41o- cells.

## DISCUSSION

In the current study, we have shown that HO-1 expression is reduced in vitro in CF airway epithelial cells (CFBE41o- cells and lungs of CFTR−/− mice) compared with normal human bronchial epithelial cells (16HBE14o- cells and lungs of wild-type mice). We have also demonstrated that impaired TLR4 in CF might lead to decreased HO-1 expression. We also found a correlation between increased iron concentrations and decreased HO-1 expression in CF, which seem to be interdependent. We further demonstrated that increased iron in CFBE41o- impairs the stability of HIF-1α, thereby leading to altered cellular response to hypoxia that includes reduced HO-1 expression.

Previous studies revealed that TLR4 expression is reduced in CF airway epithelial cells (CFBE41o- and IB-3), leading to a reduced MyD88-related and Toll/IL-1 receptor domain-containing adaptor-inducing IFN-β (TRIF)-related activation of IL-6/IL-8 and type I interferons/interferon-γ-inducible protein-10 (20, 22). These reports were supported by the in vivo findings using bronchial biopsies, where TLR4 expression was significantly reduced in the airway epithelium of patients with CF ($n$ = 7) compared with control subjects ($n$ = 6) (16). However, the underlying mechanisms are still unclear. TLR4-

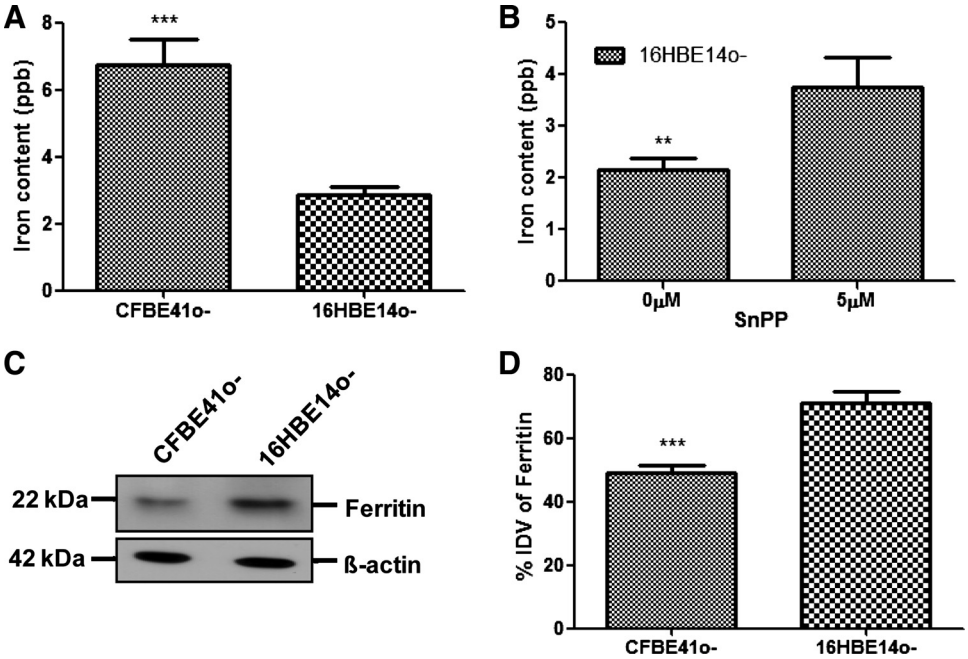


Fig. 4. Iron quantification in CFBE41o- and 16HBE14o- cell lines. *A*: quantification of iron in CFBE41o- and 16HBE14o- cells using inductively coupled plasma mass spectrometry (ICP-MS). PPB refers to parts per billion. Error bars were obtained from 3 independent standard deviations. Data represent means ± SE, and asterisks indicate statistical significance determined by Student's *t*-test (***$P < 0.05$). Significance is relative to 16HBE14o- cell line. *B*: quantification of iron in 16HBE14o- cells treated with 5 μg/ml of HO-1 inhibitor (tin protoporphyrin, SnPP) overnight using ICP-MS. Untreated samples served as control group. Data represent means ± SE, and asterisks indicate statistical significance determined by Student's *t*-test (**$P < 0.001$). Significance is relative to untreated control. *C*: representative Western blot image of ferritin expression in CFBE41o- and 16HBE14o- cells ($n = 6$). Immunoblotting of β-actin performed as loading control. *D*: densitometry analysis of ferritin immunoblots after normalizing against β-actin (loading control). Error bars were obtained from 6 independent standard deviations. Data represent means ± SE, and asterisks indicate statistical significance determined by Student's *t*-test (***$P < 0.001$). Significance was calculated and is relative to respective 16HBE14o- cell line.

mediated HO-1 upregulation has already been reported in acute lung injury, hepatic ischemia/reperfusion, and macrophages (35, 39).

Cells with ΔF508 CFTR mutation were shown to accumulate intra- and extracellular iron. Increased iron availability was demonstrated to favor biofilm formation by *aeruginosa*, whereas iron chelators along with the antibiotic tobramycin have been shown to impair the biofilm formation (30). Iron accumulation in HO-1-deficient condition leading to cell death has been manifested in fibroblasts (11, 32). In addition, HO-1 requires the coexpression of ferritin, another antioxidant for sequestering prooxidant labile Fe released from Heme (27, 32). We have already shown that serine proteases degrade mucins present in the airway secretions of CF (18). Moreover, increased expression of human neutrophil elastase, a serine protease, was shown to increased intracellular accumulation of nonheme iron via degradation of ferritin (12). Therefore, the abnormal iron accumulation in CFBE41o- cells and in HO-1-inhibited 16HBE41o- cells and the decreased ferritin expression in CF evidenced in our study are in concordance with the above-mentioned literature. This suggests the pivotal role of HO-1 in iron homeostasis.

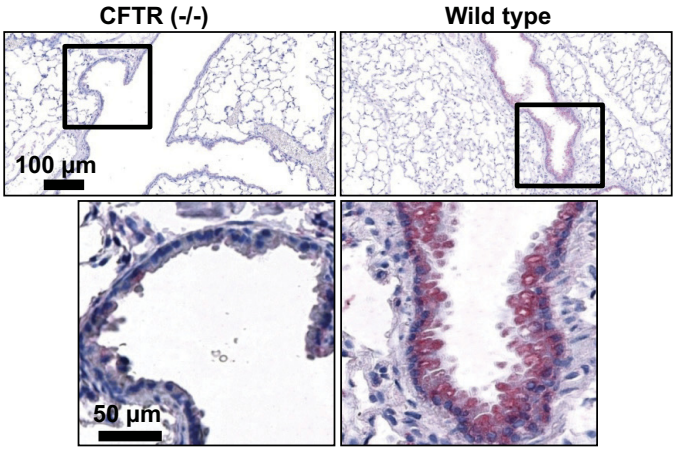


Fig. 5. Ferritin staining in CFTR (−/−) and wild-type mice lungs. *A*: representative images of immunohistochemical analysis of ferritin (pink) on paraffin sections of CFTR−/− ($n = 6$) and wild-type mice ($n = 6$). Nuclei are stained blue.

In CF, repeated bacterial insults and the inability to clear the bacterial infections result in persistent mucus accumulation on the epithelial linings, making the gas exchange extremely difficult. This, in addition to increased iron-mediated reactive oxygen species, alters the microenvironment of the airway epithelial cells in chronic CF, thereby leading to low partial pressure of oxygen inside the lungs and thus hypoxia (6, 40). We hypothesized that LPS and the tissue hypoxia would elicit a stronger hypoxic response in CF. In contrast, we observed less hypoxic response (HIF-1α) in CF probably attributable to defective TLR4 and altered iron homeostasis (Fig. 8). Under hypoxia and LPS treatment, the HO-1 expression was much less in CFBE41o- cells than in 16HBE14o- cells. HIF-1α mediates the transcriptional activation of HO-1 by binding to the HRE (21) located within HO-1 promoter (28). Thus decreased HIF-1α in CF leads to reduced expression of HO-1

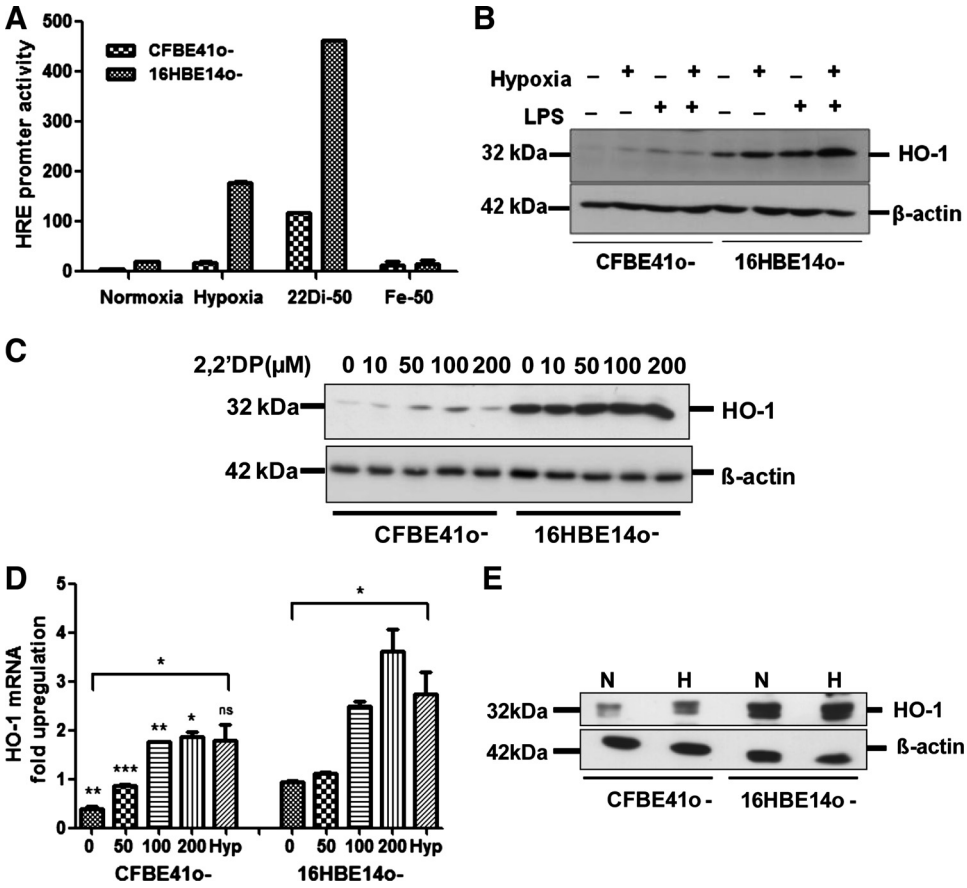


Fig. 6. Hypoxic response and HO-1 quantification in CFBE41o- and 16HBE14o- cells. *A*: hypoxia response element (HRE) luciferase reporter gene analysis in CFBE41o- and 16HBE14o- cells under normoxia, hypoxia, and upon treatment with iron chelator, 2,2′-dipyridyl (2,2′-DP) (50 μM), and iron excess $Fe^{2+}$ (50 μM). Fold expression is represented after normalization of the expression values with internal control ($n = 4$). *B*: Western blot image of HO-1 expression in CFBE41o- and 16HBE14o- cells under normoxia and hypoxia overnight, with or without LPS (2 μg/ml) overnight. *C*: Western blot image of HO-1 expression in CFBE41o- and 16HBE14o- cells upon dose-dependent treatment of iron chelator (2,2′-DP) overnight. *D*: quantification of HO-1 mRNA in CFBE41o- and 16HBE14o- cells upon stimulation with increasing concentrations of 2,2′-DP (50, 100, and 200 μg/ml) and 0.1% hypoxia using real-time PCR. HO-1 transcripts of CFBE41o- and 16HBE14o- were normalized with their respective β-actin transcripts (internal control). HO-1/β-actin of 16HBE14o- were compared with HO-1/β-actin from CFBE41o-. Error bars were obtained from 4 independent standard deviations. Significance was calculated using Student's unpaired *t*-test, $*P < 0.05$ and is relative to respective treatment for each cell line unless otherwise indicated with bars. $**P < 0.01$, $***P < 0.001$; ns, not significant. *E*: Western blot detection of HO-1 in CFBE41o- and 16HBE14o- cells upon incubation at 0.1% hypoxia. N, normoxia; H, hypoxia.

under hypoxia. HIF-1α has also been demonstrated to enhance the expression of TLR4 in macrophages, BV-2 cells (microglia cells), and pancreatic cancer cells under hypoxia (10, 24, 42). In turn, LPS-mediated TLR4 signaling has been proven to induce HIF-1α in human gingival fibroblasts and dendritic cells, even under normoxia (29, 36). TLR activation and hypoxia were suggested to recruit distinct signaling mechanisms to stabilize HIF-1α (19). In this study, defective TLR4 in CF might contribute to decreased HIF-1α under normoxia and vice versa under hypoxia.

Under normoxia, reduced TLR4 surface expression might lead to decreased HIF-1α expression. Contrastingly, under hypoxia also there is an impaired HIF-1α expression in CF.

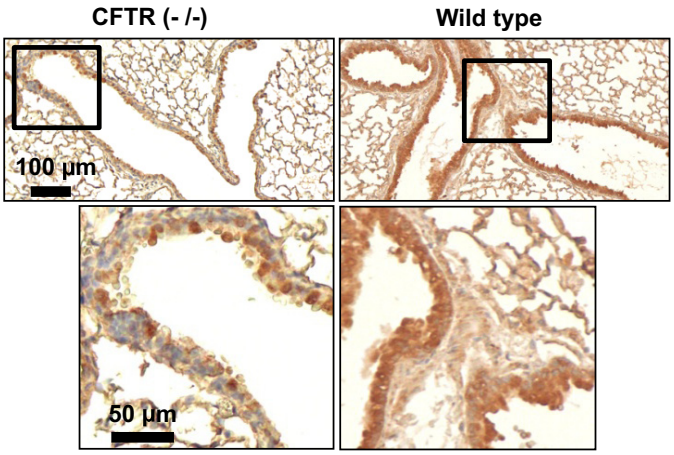


Fig. 7. Hypoxia-inducible factor (HIF)-1α staining in CFTR (−/−) and wild-type mice lungs. Immunohistochemical analysis of HIF-1α (brown) on paraffin sections of CFTR (−/−) ($n = 6$) and wild-type mice ($n = 6$). Nuclei are stained blue.

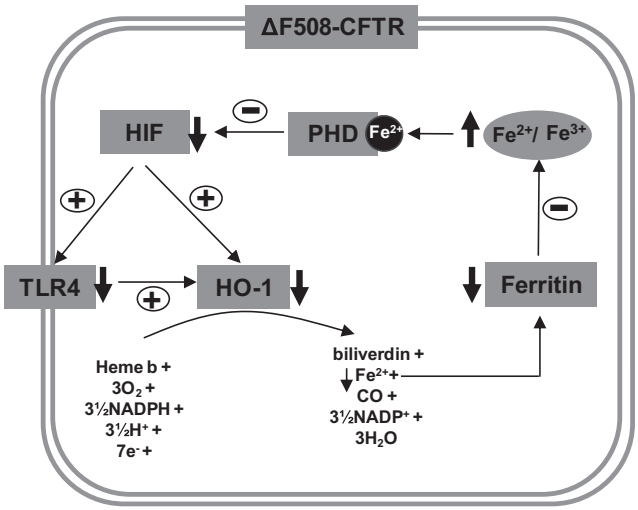


Fig. 8. Schematic representation of altered cellular homeostasis in CF. Functional components for iron sensing, iron storage, and iron homeostasis are represented in gray boxes. Upregulation or downregulation of the respective targets are indicated with block arrows. Positive or negative regulation of genes are indicated as (+) and (−), respectively. Fe, iron; PHD, prolyl hydroxylase domain.

The activity of HIF depends on the stabilization of HIF-1α, which in turn depends on prolyl hydroxylase domain (PHD) enzymes. In the presence of $O_2$, $Fe^{2+}$, and 2-oxoglutarate, PHD hydroxylates proline residues of HIF-1α, leading to polyubiquitination and proteasomal degradation of HIF-1α. Inactivation of PHD increases the half-life of HIF-1α and in turn HIF-1. $Fe^{2+}$ is present at the active site of PHDs. $Fe^{2+}$ was demonstrated to be essential for the activity of PHDs. By reducing the availability of $Fe^{2+}$ using iron chelators or by substituting $Fe^{2+}$ with other metal ions such as $Co^{2+}$, $Ni^{2+}$, and $Mn^{2+}$, one can abrogate the activity of PHDs (23). Thus we hypothesized that excess intracellular iron present in CF could possibly be leading to HIF-1α destabilization via $Fe^{2+}$-stabilized PHDs (Fig. 7). For hypoxia-independent HIF-1α activation, recent studies deploy bacterial siderophores for iron deprivation of host cells (15). A similar strategy is to employ iron chelators, namely 2,2′-DP or desferroxamine (25), to inhibit $Fe^{2+}$-dependent PHD activation and HIF-1α stabilization. In our investigation, we used 2,2′-DP treatment to remove the excess of intracellular iron in CFBE41o- and found an improved HIF-1α response in CF compared with normal condition. In the iron-repleted state (excess $Fe^{2+}$ added to the media), a complete shutdown of HIF-1α expression was observed in both cell lines (CFBE41o- and 16HBE14o-). A slight increase in HO-1 in CF was also evidenced upon 2,2′-DP treatment. These results suggest that excess of $Fe^{2+}$ in CF might interfere with stability and activity of HIF-1α expression under hypoxia. Thus we speculate that treatment with iron chelators in CF might, not only curb *Pseudomonas aeruginosa* biofilm formation by restricting the bioavailability of iron directly, but also enhance the activity of HIF-1 and thereby HO-1 expression under tissue hypoxia.

In conclusion, reduced TLR4 surface expression in addition to the altered iron homeostasis and hypoxic response in CF contributes to an ineffective anti-inflammatory and antioxidant response via HO-1 in vitro. The defect in production of this major antioxidant in vivo might lead to exaggerated airway inflammation. Thus the current in vitro data give us an idea that treatments targeting the induction of HO-1 could be potentially successful in overcoming the increasing iron load and decrease the oxidative stress in CF. Further studies are needed to confirm the alterations in the above-mentioned pathways in the primary airway epithelial cells from CFTR knockout mice or nasal epithelial cells obtained from patients with CF, which will support our findings and might play a role in pathophysiology of CF disease.

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## DISCLOSURES

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

## AUTHOR CONTRIBUTIONS

Author contributions: S.C. and M.O.H. conception and design of research; S.C., S.V., V.G., P.M., A.S., and G.S. performed experiments; S.C., V.G., P.M., A.M., A.S., A.G., B.T.S., and B.T. analyzed data; S.C., V.G., P.M., A.M., A.S., A.G., B.T.S., B.T., and M.O.H. interpreted results of experiments; S.C. and P.M. prepared figures; S.C., P.M., A.M., B.T.S., B.T., and M.O.H. drafted manuscript; S.C., P.M., A.M., A.G., B.T.S., B.T., and M.O.H. edited and revised manuscript; S.C., S.V., P.M., A.M., A.S., A.G., B.T.S., B.T., and M.O.H. approved final version of manuscript.

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