Short communication

Impaired expression of hypoxia-inducible factor-1α in cystic fibrosis airway epithelial cells – A role for HIF-1 in the pathophysiology of CF?

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Abstract

The continuous infection–inflammation cycle plays a crucial role in the progression of cystic fibrosis (CF) disease. This noxious loop can be aggravated by a reduced partial pressure of oxygen in the blood, hypoxemia, present in CF patients. These interconnected factors, hypoxia, inflammation and infection, by stabilizing the hypoxia-inducible factor-1α (HIF-1α) protein subunit, are able to activate the transcription factor HIF-1. To date, data investigating the potential role of HIF-1 in CF are scarce. Our results demonstrated that HIF-1α protein expression was altered in CF-affected compared to CFTR-corrected airway epithelial cells in unsimulated and simulated hypoxic conditions. In contrast, when CF-affected cells were infected with *Pseudomonas aeruginosa*, HIF-1α was more stabilized compared to CFTR-corrected cells. As HIF-1 is linked with an efficient immune response and pulmonary complications in cystic fibrosis, this difference in HIF-1α protein levels could have an impact in the CF pathology and the persistence of *P. aeruginosa* infection.

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1. Introduction

The deterioration of pulmonary function resulting from the infection–inflammation cycle experienced by patients with cystic fibrosis (CF) can lead to hypoxemia, reduced partial pressure of oxygen in blood. More severe forms of hypoxia can engender clinical complications such as pulmonary hypertension (1). Furthermore, hypoxia (reduced oxygen availability) exacerbates the inflammatory responses (2) and as such could contribute to a decline in pulmonary function in CF patients. Finally, hypoxic gradients found in thick mucus on CF epithelial surfaces appear to promote biofilm associated *P. aeruginosa* infection (3) and consequently hypoxia could contribute to long-term survival of *P. aeruginosa* in CF lungs and to its associated chronic airway damage.

Molecular and cellular responses to hypoxia are mediated by the transcription factor hypoxia-inducible factor-1 (HIF-1). Target genes of HIF-1 regulate several biological processes including cell survival, angiogenesis and energy metabolism that allow the cell, tissue and organism to adapt to cellular stress caused by oxygen deficiency (4). HIF-1 is composed of two protein subunits, HIF-1α and HIF-1β (5), but its transcriptional activity is dependent on the stabilization of HIF-1α. While HIF-1β subunit is constitutively expressed in the cells, expression of HIF-1α protein is regulated at a post-translational level by prolyl hydroxylase domain (PHD) proteins, through the hydroxylation of HIF-1α and subsequent proteasomal degradation (6). The activity of PHD enzymes is inhibited by low oxygen tension, permitting HIF-1α protein stabilization during hypoxia. In addition to hypoxia, other factors modulate PHD activities, and consequently adjust HIF-1α expression levels, such as iron and divalent metal ions (7) and reactive species oxygen (ROS) (8). In addition, in infected tissues, where the microenvironment is hypoxic, increasing evidence suggests a key role for HIF-1 signalling, leading to optimization of innate immunity, control of the pro-inflammatory response and bacterial killing (9).

While HIF-1 has been shown to regulate CFTR expression (10), there is no data available on the effect of knocking out CFTR on HIF-1α stabilization, or on the role of the HIF-1 pathway in...
CF, where inflammation, infection and hypoxia play a major role in the progression of the disease.

2. Material and methods

All reagents were obtained from Sigma Aldrich, unless stated otherwise.

2.1. Cell culture

IB3-1 (ATCC CRL-2777) is a bronchial epithelial cell line derived from a CF patient with CFTR ΔF508/W1282X alleles (CF-affected cells). S9 cells (ATCC CRL-2778) are IB3-1 cells corrected for CFTR expression by transfection with wild-type adeno-associated viral CFTR (AAVCFTR) (CFTR-corrected cells). Both cell lines were purchased from the American type Culture Collection (ATCC, LGC Standards, UK). Cells were cultured on bovine serum albumin–collagen–fibronectin-coated flasks using LHC-8 medium (Invitrogen, Ireland) supplemented with 5% foetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin (Invitrogen, Ireland) under a 5% CO₂ humidified atmosphere at 37 °C.

2.2. Hypoxia-mimetic condition and infection procedure

Cells at 80% of confluence were maintained in LHC-8 medium without FBS and antibiotics. For hypoxia-mimetic condition, cells were treated with the iron chelator and PHD inhibitor, 2,2'-dipyridil (DP) at a concentration of 100 μM for 24 h. For the infection procedure, Pseudomonas aeruginosa PAO1 originally obtained from B. Iglewski was cultured aerobically for 16–18 h in LB media at 37 °C under agitation and subcultured in LHC-8 medium for an additional 3 h. S9 and IB3-1 cells were infected at an initial multiplicity of infection (MOI) of 50 to 1 cell. Then, cells were collected after 1.5, 3 and 6 h of infection. MOI were checked by plating serial dilutions of PAO1 onto LB plates.

2.3. Western blotting

Ten micrograms of proteins was resolved on 8% SDS–PAGE and transferred to nitrocellulose membrane. The following antibodies were used: mouse anti-human HIF-1α (610958, clone 54, BD Transduction Laboratories) and mouse anti-β-Actin (A5441, clone AC-15, Sigma Aldrich). Detection was performed using ECL chemiluminescence (Fisher scientific, Pierce). Band intensities were determined by densitometry using GeneTools software (SynGene, Cambridge, UK).

2.4. Statistical analysis

Three independent biological replicates were performed for all experiments described in this manuscript. The band intensity of HIF-1α was normalized against that of β-Actin. Results were expressed as the mean HIF-1α ratio ± standard deviation (SD) (n = 3) and analysed relative to control ratios, given the arbitrary value of 1. Statistical analysis was performed using Student’s t-test. Differences were considered significant if the P-value was ≤ 0.05.

3. Results

To investigate whether HIF-1 complex is altered in CF-affected lung epithelial cells, we compared the HIF-1α protein levels between CF-affected (IB3-1) and CFTR-corrected (S9) bronchial epithelial cell lines. Interestingly, CF-affected cells contained significantly 40% less HIF-1α protein than cells corrected to express CFTR (mean HIF-1α ratio: 0.63±0.031; P-value ≤ 0.01) (Fig. 1). As there was no difference in HIF-1α transcript levels between cell types (data not shown), the decrease in protein levels may be due to increased HIF-1α degradation by PHD enzymes in CF-affected cells, possibly as a result of reduced levels of factors involved in the inhibition of PHD activity. Alternatively, lack of dysfunction of CFTR protein could influence HIF-1α expression. Nonetheless, it is possible that this lower level of HIF-1α protein in CF-affected cells could interfere with the response to hypoxemia and infection in CF patients. This led us to investigate the HIF-1α response in CF-affected cells during pathophysiological conditions. Therefore, cells were exposed to a hypoxia-mimetic agent, 2,2'-dipyridilid (DP) at a concentration of 100 μM for 24 h. DP treatment resulted in a significant induction of HIF-1α protein levels in both cell lines, but with a significantly higher level of induction in CFTR-corrected (mean HIF-1α ratio: 3.45±0.25; P-value ≤ 0.01) compared to CF-affected cells (mean HIF-1α ratio: 1.83±0.22; P-value ≤ 0.05) (Fig. 2). This data suggests that the presence of the
levels in CF patients have been correlated with enhanced HIF-1 binding to the VEGF promoter (21), suggesting that this may be linked to a reduced expression of VEGF (vascular endothelial growth factor), which plays an important role in the development of primary lung graft dysfunction (PGD) (20). Elevated VEGF levels found in serum of CF patients have been correlated with enhanced HIF-1 binding to the VEGF promoter (21), suggesting that this may be linked to a higher susceptibility to PGD in CF patients. Furthermore, aberrant activation of the HIF-1-VEGF pathway has been linked to the development of pulmonary hypertension (22). Indeed, pulmonary hypertension induced by hypoxia was delayed in heterozygous CFTR-deficient mice (23). However, to date, the role of HIF-1 in pulmonary hypertension in the context of the CF pathology has not been studied.

Finally, adenosine has been implicated in tissue protection during hypoxic conditions and HIF-1, by repressing equilibrative

4. Discussion

Evidence from this study indicates a new role for HIF-1 in cystic fibrosis pathology, as suggested by the reduction of HIF-1α levels in the absence of a functional CFTR protein in airway epithelial cells. HIF-1 is known to be regulated by a complex regulatory network, including both positive and negative factors, which regulate HIF-1α expression through a variety of mechanisms including transcription, translation, post-translational modification, protein–protein interaction and degradation (11). As such, multiple factors associated with CF pathology could contribute to modulation of HIF-1α levels. For instance, CF patients are known to have reduced NO levels (12) and NO is known to play a role in the stability of the HIF-1α subunit (13). On the other hand, known inducers of HIF-1α stabilization, such as NF-kB and ROS (2, 8) have been shown to be increased in CF cells (14). This fine balance between positive and negative factors resulted in reduced HIF-1α levels in CF-affected cells. Furthermore, the fact that HIF-1α stabilization was rapidly induced in response to P. aeruginosa suggests that infection was able to overcome the effect of the CFTR mutation on HIF-1α levels and/or a distinct pathway of stabilization was employed. These differences in levels of HIF-1α could have consequences for the health of CF patients as many HIF-1 target genes could be linked to the CF pathology.

By driving expression of genes involved in host immunity and inflammation, studies with HIF-1α knockout mice revealed that activation of HIF-1 after pathogen interaction (Group A Streptococcus, Yersinia enterocolitica or P. aeruginosa) is essential for a full inflammatory response in the host (15–17). However, as we know, CF-affected cells have imbalanced inflammatory responses during infection. Thus, increased HIF-1α stabilization following P. aeruginosa infection could be associated with excessive and deleterious inflammation. Furthermore, a key link to HIF-1 and P. aeruginosa pathogenesis may be its ability to facilitate the induction of PA-I Lectin (PA-IL) in P. aeruginosa upon exposure to epithelial cells (18). PA-IL is a potent barrier-dysregulating protein and has been shown to facilitate exotoxin A crossing the intestinal epithelial barrier resulting in lethal gut derived sepsis in mice (19). PA-IL has also been shown to be cytotoxic to respiratory epithelial cells and may contribute to epithelial damage during P. aeruginosa respiratory infections.

Moreover, HIF-1 regulates the critical pro-angiogenic factor VEGF (vascular endothelial growth factor), which plays an important role in the development of primary lung graft dysfunction (PGD) (20). Elevated VEGF levels found in serum of CF patients have been correlated with enhanced HIF-1 binding to the VEGF promoter (21), suggesting that this may be linked to a higher susceptibility to PGD in CF patients. Furthermore, aberrant activation of the HIF-1-VEGF pathway has been linked to the development of pulmonary hypertension (22). Indeed, pulmonary hypertension induced by hypoxia was delayed in heterozygous deficient Hif-1α+/- mice (23). However, to date, the role of HIF-1 in pulmonary hypertension in the context of the CF pathology has not been studied.
nucleoside transporter 1 (ENT1) expression, increases extracellular adenosine half-life during hypoxia (24). Since adenosine is an important regulator of airway surface liquid volume (25) and serves a protective role during hypoxia and inflammation (24), this nucleoside could play an essential role in the progression of the disease. Concerning another HIF-1 target gene potentially involved in CF pathology, the anti-inflammatory protein netrin-1 seems to be a good candidate as netrin-1 has been shown to reduce pulmonary inflammation during the initial stages of acute lung injury (26) and HIF-1α-dependent induction of netrin-1 attenuates hypoxia-elicited inflammation at mucosal surfaces (27).

In conclusion, the potential role of HIF-1 in the pathophysiology of CF is raised. This study demonstrates that lower levels of HIF-1α protein are found in CF-affected versus CFTR-corrected airway epithelial cells, independent of hypoxemia, but with a hyper-stabilization of this protein in response of P. aeruginosa infection. These differences in the levels of HIF-1α protein could impact on CF pathology as HIF-1 target genes play a pivotal role during hypoxia, inflammation and infection. Therefore, further work is warranted to understand the mechanism underlying the role of HIF-1 in the context of cystic fibrosis pathology.

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