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RESEARCH ARTICLE

Effect of hypoxia on Cystic Fibrosis Transmembrane conductance Regulator channel corrected by Elexacaftor/ Tezacaftor/ Ivacaftor

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ABSTRACT

The accumulation of mucus resulting from the obstruction of bronchi of cystic fibrosis (CF) patients, induces a reduction of the oxygen (O_2) pressure and produces a hypoxic environment for the epithelial cells of the lungs. Our study aims to better characterize the impact of hypoxia on CFTR function in the pathophysiological context of cystic fibrosis.

We used Human airway epithelial cells from two CF donors and human bronchial epithelial cell lines non-CF and CF, grown and expended in normoxia (21% O_2) and then switched to hypoxia (1% O_2) for 2 to 24 hours. Cells were treated by dimethyl sulfoxide or Elexacaftor/ Tezacaftor/Ivacaftor for 24 hours.

We show that the peak of Hypoxia Inducible Factor 1α is reached in a range of 4 to 6 hours post-hypoxia induction. We also demonstrate that the global amount of ETI corrected F508del-CFTR is significantly decreased after 24 hours of hypoxia. A decreased ETI corrected F508del-CFTR activity was recorded by both patch-clamp and Ussing chamber recordings.

Our results show that hypoxia, despite the effectiveness of Elexacaftor/ Tezacaftor/Ivacaftor correction, impacts the downstream effects of the F508del mutation, which suggests that oxygen availability in the lungs is a factor to take into account for the administration of Trikafta to patients.

Keywords: HIF-1α, cystic fibrosis, human bronchial cells, Trikafta

Introduction

Cystic fibrosis (CF), one of the most common autosomal recessive diseases is caused by mutations of the *CFTR* (Cystic Fibrosis Transmembrane conductance Regulator) gene coding for a chloride channel[1]. It is expressed in epithelial cells and controls bicarbonate, water, and chloride transports. The affected protein has, among other things, an altered transport function leading to the malfunction of various exocrine glands, especially in the lungs[2]. The most common mutation F508del-CFTR, corresponding to the deletion of a phenylalanine at position 508 promotes the accumulation of a viscous mucus in the airways[3]. Obstruction of bronchi and alveoli makes the body's oxygenation less efficient. Respiratory failure is commonly described as the inability of the lungs to realize haematosis, a mandatory for aerobic metabolism at the tissue level[4].

Hypoxia is defined as a low oxygen (O_2) level in body tissue and can cause symptoms such as headache, confusion, difficulty to breath or blueish skin. At a cellular level the hypoxia regulation central point is the Hypoxia Inducible Factor 1 alpha (HIF-1α). This protein was discovered in 1995[5] and the characterization of its mechanism of action showed that in normoxia, it is hydroxylated continuously, and conducted to the Von Hippel Lindau (VHL) pathway leading to its degradation by the proteasome complex. However, if the O_2 level drops, this hydroxylation is ineffective allowing HIF-1α to translocate into the nucleus to form a complex with HIF-1β[6]. The dimer is considered as a transcription factor able to target some specific regions on gene promoters called Hypoxia Response Elements (HRE). Once the interaction is made, it triggers the transcription of multiple genes to respond to hypoxia such as angiogenesis, metabolism, or erythropoiesis. Among all these genes, some encode for ion pumps or channels such as the Epithelial Sodium Channel (ENaC), the Na^+/K^+ ATP_{ase} pump, Transient Receptor Potentials (TRPs) or CFTR[7].

Patient's lungs with muco-obstructed diseases, such as CF, display micro-environment niches notably characterized by hypoxia. In 2002, Worlitzsch et al.[8] realized one of the rare *in-vivo* measurements of oxygen pressure (pO_2), by bronchoscopy, directly into the lung's muco-purulent masses of CF patients. They found that when the oxygen probe was located into the non-obstructed region of the bronchial lumen, the average pO_2 value reached 180 millimeters of mercury (mmHg; 23.6% O_2) (consistent with the O_2 supplementation during the experiments). However, when the probe was inserted into the mucus layer, oxygen pressure dropped rapidly to 2.5 mmHg (0.3% O_2) indicating a hypoxia environment in which bronchial cells are exposed. This regional hypoxia is aggravated by an increased epithelial oxygen consumption most likely due to an increased sodium (Na^+) uptake (mediated by ENaC) consequence of an elevated activity of the Na^+/K^+ ATP_{ase} pump[9,10]. This can result in a vulnerability of airway epithelial cells to hypoxia.

Submerging cells in culture media was reported to trigger low oxygen pathways (HIF-1α pathways) reducing the steady level of CFTR mARN and proteins as well as the transepithelial transport related to this channel. Interestingly, in CF-affected cells, the expression of HIF-1α is impaired while the expression of WT-CFTR

by transfection restored this phenomenon. This highlights a particular link between CFTR and HIF-1α but also an altered ability to respond to hypoxia in the pathophysiological context of CF.

Elexacaftor/Tezacaftor/Ivacaftor (ETI) is the most recent tri therapy for this pathology[11,12] but its correction properties on F508del-CFTR in a hypoxia environment have not been studied yet. Moreover, few studies explored hypoxia in a CF context. Here, we evaluated the impact of hypoxia on CFTR maturation and function as it is expressed in respiratory epithelium where it interacts with multiple entities sensitive to oxygen level such as ENaC or TRPs.

Materials & Methods

Cell culture

Primary cultures were grown from human airway epithelial (hAE) cells (F508del homozygous cells) obtained after lung transplantation (Foch hospital, Suresnes, France). Collecting information and protocols can be found elsewhere[13]. HAE primary cultures were seeded in dishes with Pneumacult™-Ex medium (StemCell Technologies, France) as a proliferation medium.

The human bronchial epithelial cell lines non-CF (CFBE41o- wild type CFTR cells) and CF (CFBE41o- F508del-CFTR cells), provided by D. Gruenert, (University of California, San Francisco CA, USA) were grown and maintained in Eagle's minimum essential medium containing non-essential amino acids (Gibco, USA) supplemented with 10% foetal bovine serum (FBS) (Eurobio, France), 2mM L-glutamine, 1% penicillin/streptomycin (Sigma Aldrich, Germany) and 5µg/mL puromycin as selective antibiotic. All cells were grown at 37°C in a 5% CO_2 - 95% air atmosphere and media were changed every two days.

3D Cell culture

Once cells confluency reached 80%, they were dissociated and implemented on 1.12 cm^2 permeable snapwell inserts (Corning; Fischer Scientific, USA) coated with type IV collagen at $16 µg.cm^{-2}$ (hAE; 2.10^5 cells/well) or human fibronectin (CFBE; 5.10^5 cells/well) (Sigma Aldrich, Germany) at $5 µg.cm^{-2}$. After reaching confluency (2 days post seeding), media were replaced by Pneumacult™-ALI (StemCell Technologies, France)[14] for hAE or renewed with Eagle's minimum essential medium without puromycin for CFBE. At this time, cells were switched from liquid-liquid (L/L) to air-liquid (ALI) conditions allowing the generation of a pseudo epithelium (3D multiple cell layers).

Hypoxia conditions & modulator treatments

For all hypoxia conditions, cells and media were placed in a HypoxyLab™ (Oxford Optronix, United-Kingdom) workstation at 1% O_2. Cells expressing F508del-CFTR were treated either by dimethyl sulfoxyde (DMSO) or by ETI: Elexacaftor (corrector; 3 µM), Tezacaftor (corrector; 18 µM), Ivacaftor (potentiator; 1 µM) (MedChemExpress, USA)[15]. After 24 hours of correction, the cell media were changed by equilibrated media (1% O_2) and the treatments were renewed to maintain the correction during the hypoxia phase (between 2-24h).

Western Blotting

Cells were lysed in Pierce™ RIPA buffer (Invitrogen, USA) supplemented with a proteinase inhibitor cocktail (Roche, Germany) and Pefabloc® SC (Sigma Aldrich, Germany).

Primary cells were lysed in RIPA buffer complemented with EDTA/DTT/vanadate and proteinase inhibitor cocktail (Roche, Germany) and Pefabloc® SC (Sigma Aldrich, Germany) (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM DTT and 1 mM sodium orthovanadate). Cells were centrifuged at 10,000g for 10min and the supernatants were then collected to be quantified by bicinchoninic acid assay (BCA). A total of 30 µg of proteins were mixed with Laemmli buffer at a dilution of 1:6 qsp water. The migration was run on 7.5% SDS/PAGE gel, and then transferred onto a nitrocellulose membrane. Membranes were blocked using 3% (weight/volume) BSA diluted in Tris buffer saline (TBS) 0.05% Tween 20 before being incubated overnight at 4°C with primary antibodies (1:1000): mouse monoclonal anti-human CFTR MAB3480 (Merck Millipore, Germany), mouse monoclonal anti-human CFTR CFF 596/570/450 (provided by the Cystic Fibrosis Foundation, USA), mouse monoclonal anti-human HIF-1α 610985 (B-D Sciences, USA) and mouse monoclonal anti-human GAPDH sc-32233 (Santa Cruz, USA). Samples were incubated for a minimum of 2 hours with the corresponding horseradish peroxidase (HRP) conjugated secondary antibodies (1:10000) in saturation buffer at room temperature. Finally, membranes were incubated with chemiluminescent HRP substrate for 2 min and the signals were acquired using GeneGnome (SynGene Ozyme, France). Signal intensity was quantified using GeneTools software (SynGene Ozyme, France).

Patch-clamp experiments

CFBE cell lines expressing WT and F508del-CFTR were used for the patch-clamp experiments on an eight-channel Patchliner NPC-16 workstation (Nanion technologies, Munich, Germany). Our automated whole cell patch-clamp was run on low resistance chips (1.5-2MΩ). The CFTR-dependent current traces were obtained by holding the current at -40mV (theoretical E_{Cl}) between pulses at test potentials -80mV to 80mV with an increment of 20mV during the recording session. CFTR currents were activated by forskolin (FSK; 10 µM; Sigma Aldrich, USA) and genistein (GST; 30 µM; Sigma Aldrich, USA) and inhibited by $CFTR_{inh172}$ (10 µM; Calbio-chem, USA). For other details see [13].

Short-circuit Current (I_{sc}) Measurements

The transepithelial resistances of hAE cells and F508del-CFBE cells were measured with a Millicell-ERS voltmeter-ohmmeter (Merck Millipore, Germany) after the Ussing chamber experiments. Other details can be found in[13].

Statistics

Data presented are expressed as mean ± SEM of n observations. Before using a parametric or non-parametric test, samples were checked for normality using the Shapiro–Wilk normality test. Statistical significance was determined using the Unpaired t-test or Mann-Whitney test on GraphPad version 8.0 (GraphPad Software, USA). Differences were accepted to indicate statistical significance with a P less than 0.05. Statistical significances are presented as follows: * = $p<0.05$, ** = $p<0.01$.

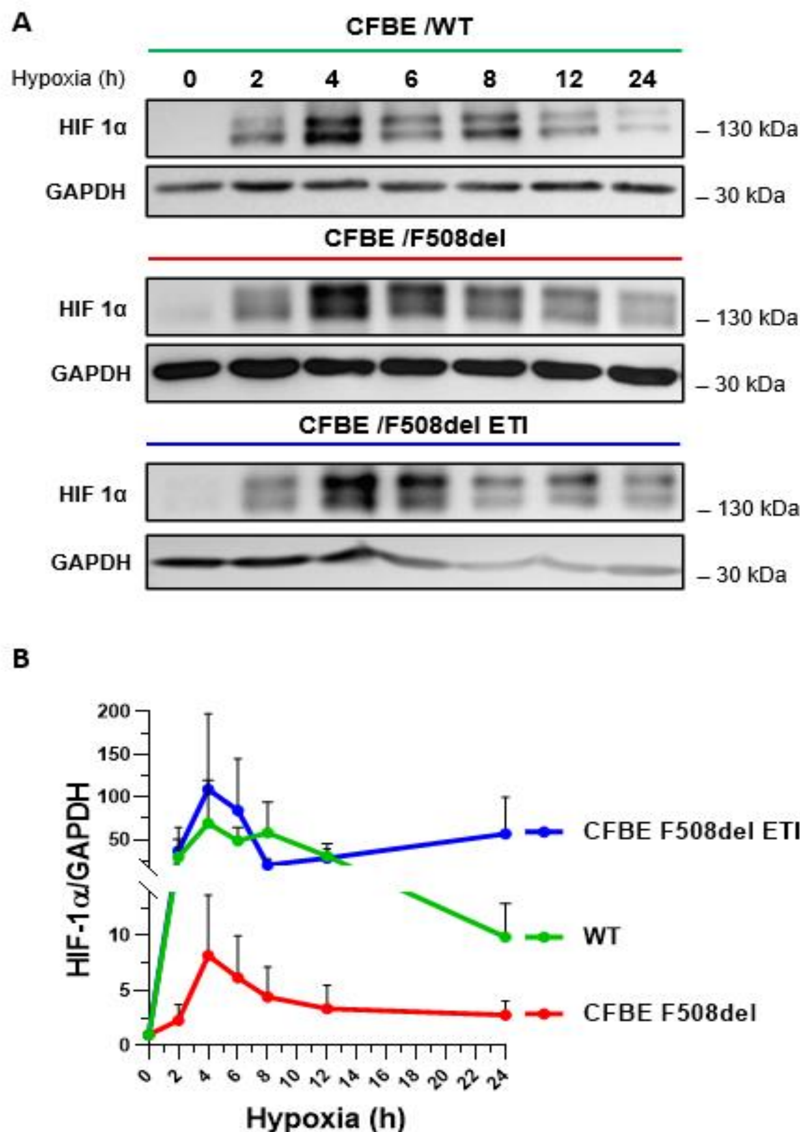
Results

HIF-1α accumulates shortly in CFBE cells in a 1% O_2 atmosphere

First, we performed western blot experiments and we showed that HIF-1α is expressed in all

our CFBE cell models and that it accumulates quickly after the switch between normoxia and hypoxia (Fig. 1). As shown in figure 1A, it takes around 4 hours in hypoxia to reach the HIF-1α peak before it decreases overtime. Interestingly, the normalization using GAPDH as a loading control showed that HIF-1α is less accumulated in non-corrected CFBE /F508del cells compared to CFBE /WT and ETI-corrected CFBE /F508del cells (Fig. 1B).

Figure 1: HIF-1α accumulation depending on hypoxia time: (A) Immunoblots showing HIF-1α protein for CFBE/WT, /F508del and /F508del corrected by ETI. **(B)** Corresponding quantification of HIF-1α accumulation over time in CFBE/WT, /F508del and /F508del corrected by ETI. Results are expressed in mean ± SEM (n ≥ 3).

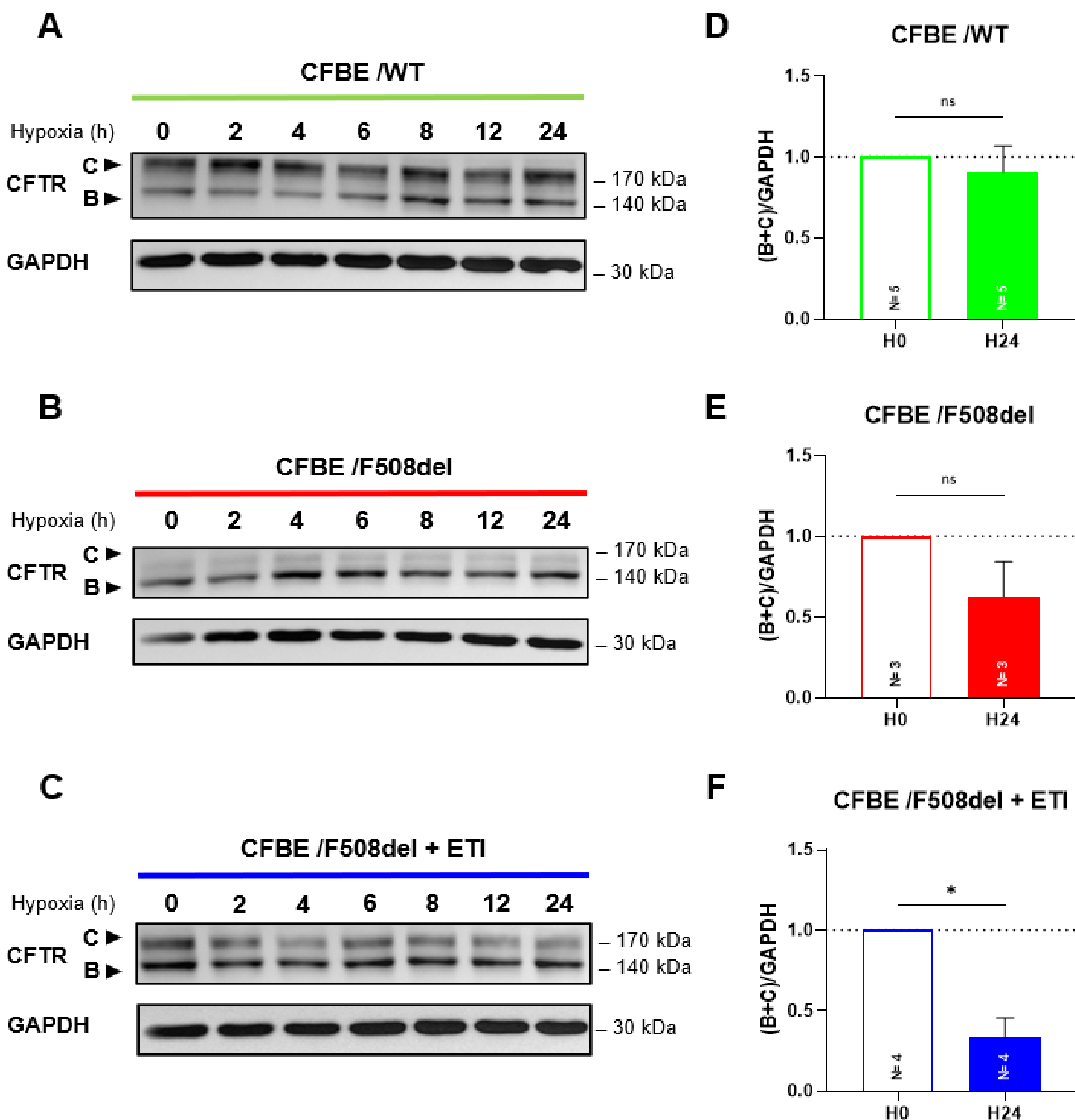


Hypoxia alters the total quantity of F508del-CFTR in CFBE

To assess whether CFTR is impacted or not by hypoxia and the accumulation of HIF-1α protein, we studied the two forms of CFTR named B (core-glycosylated non-mature CFTR) and C (mature glycosylated CFTR) forms. We show that F508del-CFTR total quantity (bands B and C) decrease over time (Fig. 2B) while

WT-CFTR remains stable (Fig. 2 A). We determined the total quantity (band (B+C)/GAPDH) for each condition (Fig. 2 D, E and F). WT-CFTR is not impacted by hypoxia in a range of 24 hours while F508del-CFTR global amount displays a trend to decrease, although not significant. However, the total amount of F508del-CFTR corrected by ETI is significantly decreased at the 24 hours timepoint (Fig. 2 C and F). The maturation of CFTR /WT, /F508del and /F508del corrected by ETI is not changed by 24 hours of hypoxia (data not shown).

Figure 2: Effects of hypoxia on the accumulation of CFTR protein in CFBE cells:
Western blots against the CFTR protein from CFBE/WT **(A)** and CFBE/F508del non corrected (NT)**(B)** or corrected by Kaftrio® (ETI)**(C)** depending of hypoxia time. Histograms of normalized ratios at normoxia (H0) and after 24 hours hypoxia (H24) for the WT **(D)**, F508del **(E)** and F508del corrected CFTR **(F)**. The (B+C)/GAPDH ratio determines the overall amount of CFTR in CFBE /WT and CFBE /F508del cells corrected or not by ETI. ns = non-significant, * = p< 0.05.



CFTR-dependent whole-cell currents are altered by hypoxia despite the correction by ETI

Next, we investigated the effect of hypoxia on the function of WT- and F508del-CFTR by recording whole-cell patch clamp currents (Fig. 3). Cells capacities values have been measured with no statistical differences in CFBE /WT (normoxia: 14.0 ± 1.47 pF (n=17) / hypoxia: 15.2 ± 3.04 pF (n=16)), CFBE /F508del (normoxia: 9.31 ± 2.64 pF (n= 4) / hypoxia: 7.9 ± 1.93 pF (n= 4)) and CFBE /F508del + ETI (normoxia: 12.6 ± 1.31 pF (n=19) / hypoxia: 15.6 ± 1.20 pF (n=28)). Representative whole-cell of corrected F508del-CFTR-dependent current traces in normoxia and hypoxia are shown in figure 3A at basal state, after adding the activation cocktail FSK+GST (10 µM/30 µM) and following the addition of the inhibitor $CFTR_{inh172}$ (10 µM). The current density versus voltage curves, for CFBE /WT, /F508del non corrected and corrected by ETI in normoxia and after 24 hours hypoxia, are presented in figure 3B. In figure 3C, we compare the effect of hypoxia on CFTR current density at a potential of +60mV during the activation phase (FSK+GST). These results show that WT-CFTR current is not altered after 24 hours of hypoxia, which agrees with our immunoblots. While the F508del-CFTR current is corrected by ETI, it is nevertheless altered by hypoxia with a 30% decrease of the current density in agreement with the reduced quantity of total CFTR proteins as shown in figure 3C.

Hypoxia decreases CFTR-dependent I_{sc} in CFBE expressing F508del-CFTR corrected by ETI

To confirm the effect of hypoxia on CFTR-dependent current, we recorded F508del-CFTR dependent I_{sc} with cells treated or not by ETI and with or without 24 hours of 3D culture in hypoxia. The resistances values obtained after the experiments are not significantly different between normoxia and hypoxia with respectively 309.6 ± 28.7 $\Omega.cm^{-2}$ (H0; n=8) / 313.6 ± 24.4 $\Omega.cm^{-2}$ (H24; n=8) for non-corrected cells and 309.6 ± 28.7 $\Omega.cm^{-2}$ (H0; n=8) / 313.6 ± 24.4 $\Omega.cm^{-2}$ (H24; n=8) for cells corrected by ETI. We also maintained the hypoxia condition during the experiment by equilibrating our medium in hypoxia and by replacing the mixture of gases used for cell oxygenation with a 1% oxygen mixture. In these experiments, I_{sc} was stimulated by forskolin (1 µM) and inhibited by $CFTR_{inh172}$ (10 µM) as shown in figure 4A. Pre-activation reflects the constitutive CFTR dependent I_{sc} restored by ETI, as shown previously [13]. The I_{sc} activation in response to forskolin, results in the opening of all F508del-CFTR channels and finally the use of $CFTR_{inh172}$ close all F508del-CFTR channels previously activated. Examples I_{sc} traces for F508del-CFBE non-corrected and corrected by ETI are shown in normoxia (Fig. 4B) and hypoxia (Fig. 4C). We found a significant I_{sc} decrease for pre-activation (57%), activation (51%) and inhibition of the F508del-CFTR corrected by ETI (47%) ($p<0.01$; Fig. 4D). These results taken together with immunoblots and whole-cell recordings indicate that F508del-CFTR corrected by ETI is sensitive to oxygen variation which reduces the amount of protein produced but also decreases F508del-CFTR channel activity and basal epithelial currents.

Figure 3: Effects of hypoxia on the activity of CFTR protein: (A) Example of current tracings with CFBE/F508del cells corrected by ETI. **(B)** Corresponding current density curves versus voltage stimulated by forskolin+genistein for CFBE /WT, /F508del non corrected and /F508del corrected by ETI. **(C)** Histograms of current density were obtained at +60mV for all cell types. ns= not-significant, * = p<0.05. Empty symbols= normoxia, black symbols= hypoxia.

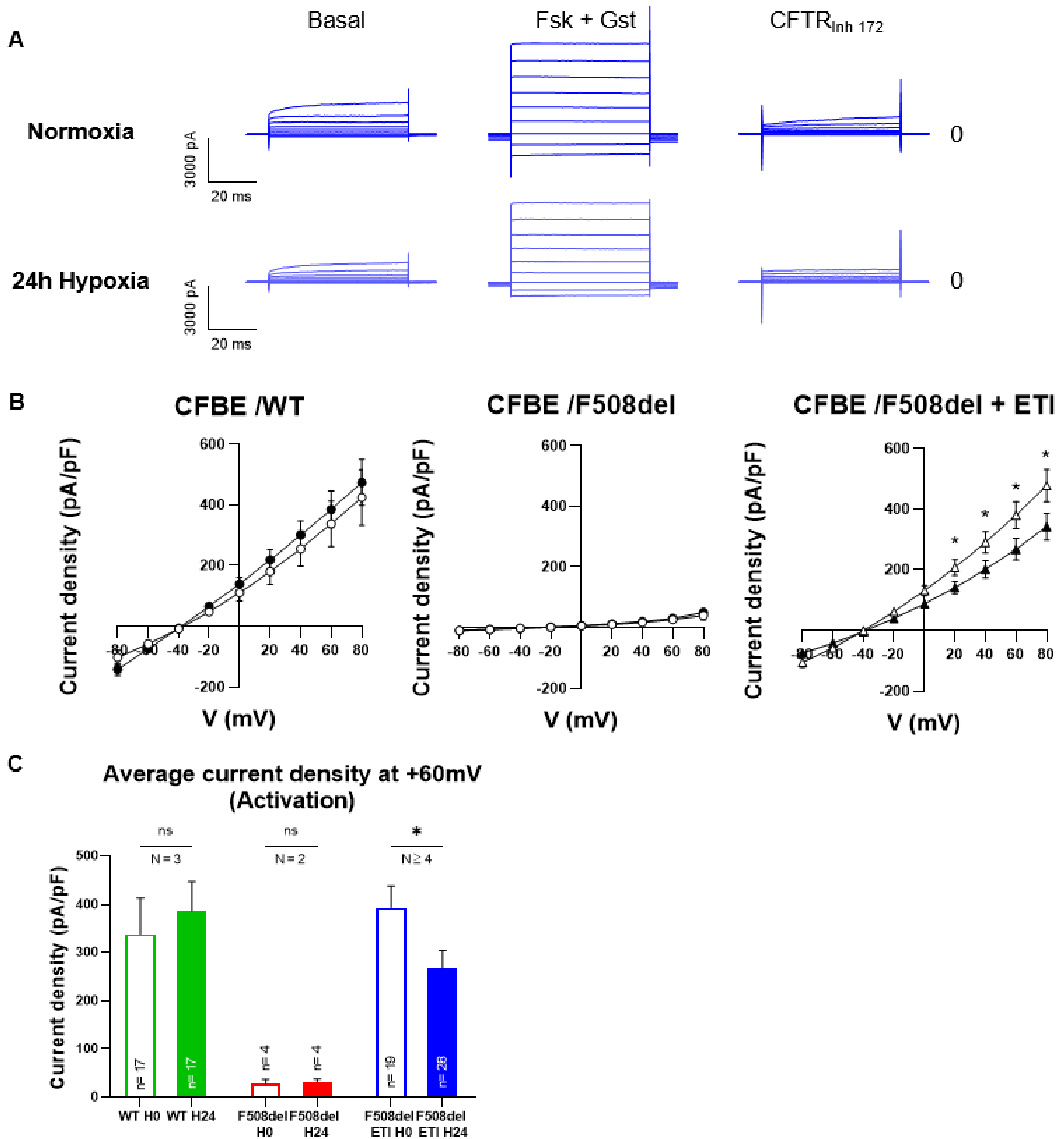
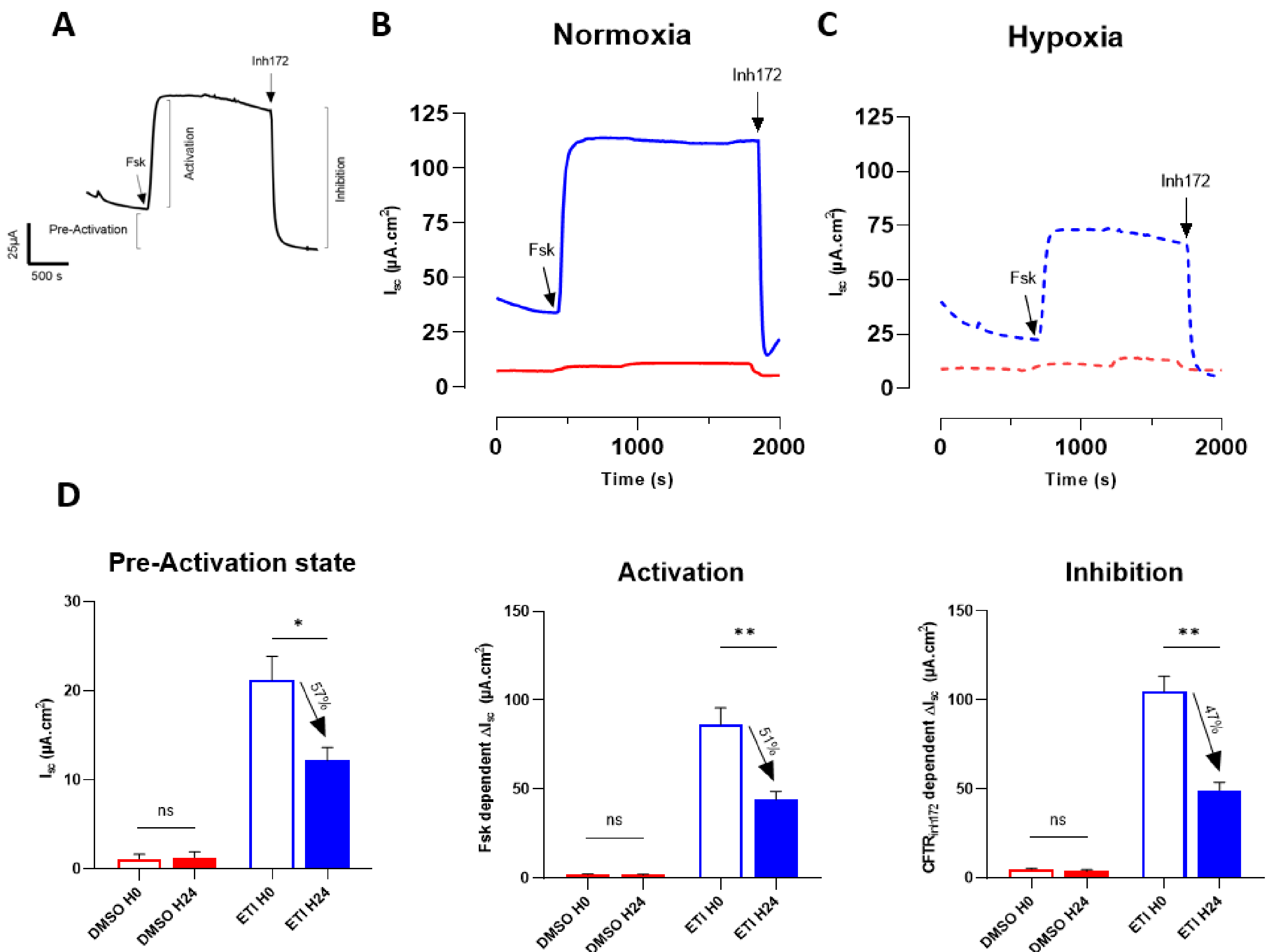


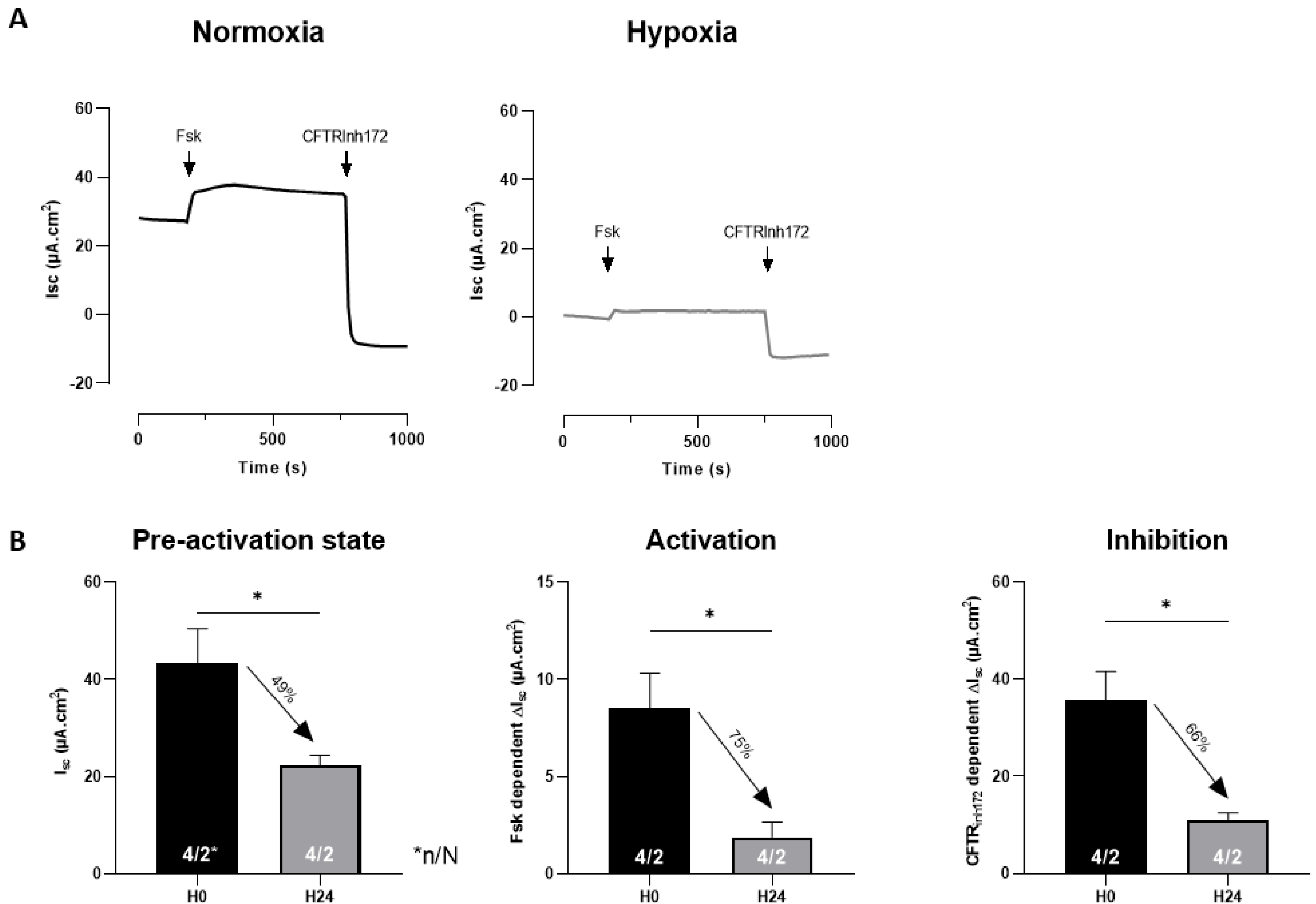
Figure 4: Effects of hypoxia on I_{sc} recordings of F508del-CFTR corrected by ETI: (A) Example of I_{sc} traces. **(B)** I_{sc} traces related to CFTR on CFBE/F508del non corrected and corrected by ETI in normoxia (H0) and **(C)** after 24 hours hypoxia (H24). I_{sc} is activated by forskolin (1 µM) and inhibited by $CFTR_{inh172}$ (10 µM). **(D)** Histogram of pre-activation, forskolin activation and inhibition I_{sc} values. ns = non-significant, *= p<0.05, ** = p<0.01.



F508del-CFTR activity is altered by 1% O_2 hypoxia in Human primary bronchial epithelial cells corrected by ETI

We investigated the effects of hypoxia on CF-patient's hAE cells corrected by ETI (Fig. 5). Hypoxia does not significantly reduce the transepithelial resistances of hAE cells after 24 hours (R_{TE}= 521 ± 53.4 Ω.cm^{-2}, H0, n=4 and 430.8 ± 33.2 Ω.cm^{-2}, H24 n=4). We recorded I_{sc} in hAE cells in normoxia or after 24 hours of hypoxia, all treated by ETI. Results presented figure 5A show that hypoxia reduces the F508del-CFTR short-circuit current and pre activated current. Similarly to what we found on CFBE cell lines, the pre-activation, activation and inhibition responses are decreased in hypoxia, respectively by 49%, 75% and 66% (Fig. 5B). Overall, the data obtained on hAE cells are coherent with the data obtained on CFBE /F508del cells, which displays a reduced activity despite the ETI correction.

Figure 5: Effects of hypoxia on maturation of CFTR and I_{sc} in human bronchial cells:
(A) I_{sc} traces related to F508del-CFTR on human primary cells non corrected and corrected by ETI in normoxia (H0) and after 24 hours hypoxia (H24). I_{sc} stimulated by forskolin (1 µM) and inhibited by $CFTR_{inh172}$ (10 µM). **(B)** Histogram of pre-activation, forskolin activation and inhibition values. ns = non-significant, * = $p<0.05$.



Discussion

Hypoxia is a topic of growing interest in physiopathology especially in the field of muco-obstructive lung diseases that include cystic fibrosis. This is why we studied here the impact of a low oxygen level on cells expressing the F508del-CFTR. In the present study, we placed F508del cells treated with ETI in a hypoxic atmosphere (24h at 1% O_2) and we showed that: i) HIF-1α accumulates from 4h of hypoxia; ii) the total amount of F508del-CFTR protein is significantly decreased; iii) the electrophysiological activity of F508del-CFTR corrected by ETI is decreased by 30%; iv) F508del-CFTR-dependent I_{sc} corrected by ETI are significantly reduced both in cell lines and primary 3D cultures from CF patients.

The results we obtained for HIF-1α accumulation are consistent with similar experiments made by *Bartoszewski et al* [16] on various human cell

lines. This experiment was necessary to ensure that in our culture conditions, CFBE cells are able to: i) react to oxygen concentration changes, ii) display the same HIF-1α kinetic over time as other cell types and iii) are not expressing this protein in normoxia as it have been shown in some human cell lines[17]. Also, it is interesting to note that similarly to *Legendre et al* [18] we found a reduced amount of HIF-1α in non-corrected CFBE /F508del compared to CFBE /WT and /F508del corrected by ETI. This suggests a relation between CFTR and HIF-1α, which has not been completely described yet. However, *Zheng et al* [19] demonstrated *in vitro* that HIF-1α was able to bind to the promoter of *CFTR-WT* in intestinal cells. On the other hand, it has been shown that the use of hypoxia mimetics, raised the level of microRNAs especially for the miR-200b [20] in HIF-1α dependent manner [21]. Thus regulation of CFTR by HIF-1α appears possible at several levels with various pathways involved, likely interrelated, and resulting in an aggravated loss of protein expression.

The significant reduction of ETI-corrected F508del-CFTR global amount observed in our immunoblots, supports the results obtained by *Guimbellot et al* in 2008[22] showing a transcription regulation of hypoxia on *CFTR via* HIF-1α, reducing the mRNA level coding for this channel. However, in our experiments, no accumulation differences were found for WT-CFTR and non-corrected F508del-CFTR after 24 hours of hypoxia. The possible explanations for that are: i) the WT-CFTR protein is more stable with a slower recycle time [23] and ii) in the absence of correction the production of the mutated protein is low. The next experiment to test the WT-CFTR stability could be to test a longer hypoxia time to 48h, where factors such as HIF-2α take over in the response to hypoxia[16].

In a recent study, *Wong et al* [24] show that chronic hypoxia (> 25 days) could impair the function of various channels such as ENaC, Calcium dependent Chloride Channel (CaCC) and CFTR in human nasal epithelial cells (hNEC) from non-CF and CF patients. Similarly to us, they studied conditions such as normoxia (21% O_2) and hypoxia (2% O_2) completed with a correction or not by ETI. Their results indicate that a chronic exposition of hNECs (30 days of differentiation) to a hypoxic atmosphere impact significantly the I_{sc} CFTR dependent especially when the cells expended in normoxia were differentiated in hypoxia. Here, we show that this alteration occurs only 24 hours after the cells were placed in low oxygen atmosphere. Moreover, we believe that our data reflect the specific effects of upregulated HIF-1α pathways on CFTR, limiting the incidence of HIF-2α, involved in response to longer hypoxia exposition[25].

Our study supports the fact that hypoxia reduces the function of CFTR at the cell membrane worsening the CF pathology by aggravating the downstream effects of the F508del mutation. Also, the beneficial effects of Trikafta® (ETI) are altered after 24 hours of hypoxia. Cells from the patient's lungs can experience such severe conditions as a pO_2 can drop close to 1%. In addition to their treatments, most of the patients receive respiratory kinesitherapy to declutter their lungs. This highlights the periodicity with which patient's pO_2 may vary. However, lungs cells are able to react to such modifications as HIF-1α pathways are activated as long as oxygen level drop in an exponential manner[26]. Finally, we can hypothesize that

Trikafta® administration requires the oxygenation conditions to be as close as possible to physiological values to ensure the efficiency of the medicine. Oxygenation level would be then a potential parameter to consider for optimizing future treatment efficiency.

Conclusion

In conclusion, our results add to the understanding of the impact of hypoxia in CF pathology by showing that i) exposing CFBE cell lines and human airways epithelial cells to 1% oxygen, during 24 hours, reduce significantly the global amount and the activity of the F508del-CFTR protein and ii) even when F508del-CFTR protein is corrected by ETI.

Conflict of Interest Statement:

The authors have no conflicts of interest to declare.

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