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The protein kinases TPL2 and EGFR contribute to ERK1/ERK2 hyper-activation in CFTRΔF508-expressing airway epithelial cells exposed to *Pseudomonas aeruginosa*

Guy Martel, Lucie Roussel, Simon Rousseau *

Meakins-Christie Laboratories, McGill University Health Centre Research Institute, Montreal, Canada

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ABSTRACT

Excessive inflammation and *Pseudomonas aeruginosa* infection are two major characteristics of cystic fibrosis (CF) lung disease. In this manuscript, we describe a novel mechanism of ERK1/ERK2 activation and CXCL8 expression in airway epithelial cells (AECs) lacking functional CFTR. In both non-CF and CF AECs, the protein kinase TPL2 is required for ERK1/ERK2 MAPK activation. However, we have found that EGFR is strongly phosphorylated in the airway epithelium of CF lung and contributes to ERK1/ERK2 MAPK activation in CF AECs exposed to *P. aeruginosa* diffusible material (PsaDM). Moreover, PsaDM stimulates the expression of the EGFR pro-ligand HB-EGF more strongly, and in a sustained manner, in CF AECs compared to non-CF cells. Finally, although both non-CF and CF AECs expresses CXCL8 in response to PsaDM, the levels of CXCL8 are higher and EGFR plays a more important role in regulating CXCL8 synthesis in CF AECs. Together, our finding shows that in addition to the TLR-mediated TPL2 activation of ERK1/ERK2, an additional pathway contributing to ERK1/ERK2 activation is triggered by infection of CF AECs: the EGFR signaling pathway. This second pathway may contribute to excessive inflammation observed in CF.

1. Introduction

In cystic fibrosis (CF), chronic infection and inflammation are associated with decreasing lung function [1,2]. *Pseudomonas aeruginosa* is the most significant pathogen in CF with up to 80% of patients eventually infected with this gram-negative bacteria [3]. Understanding the signal transduction mechanisms triggered by *P. aeruginosa* leading to inflammation is important to develop novel anti-inflammatory strategies in CF. Activation of innate immunity in response to pathogens is mediated via pattern-recognition receptors expressed by host cells, with the toll-like receptor (TLR) the best characterized family [4].

Destructive inflammation in the gut of mice is mediated by activation of the MAPK pathways [5]. In LPS-stimulated macrophages, activation of extracellular signal-regulated kinase (ERK)1/ERK2 (also known as MAPK3/MAPK1) is downstream of TLR4 via activation of the protein kinase tumor progression locus (TPL) 2, also known as MAPK kinase kinase (MAP3K)8 [6]. This pathway is important for the regulation of inflammation [6,7]. Similarly, we have shown that in the bronchial airway epithelial cell line BEAS-2B, TPL2 is also required for early ERK1/ERK2 MAPK activation and pro-inflammatory cytokine synthesis in response to TLR-activation [8].

In airway epithelial cells expressing the most common mutation leading to cystic fibrosis, CF transmembrane regulator (CFTR)ΔF508, ERK1/ERK2 are hyper activated in response to *P. aeruginosa* diffusible material (PsaDM) [9]. We wondered whether the same activation mechanisms were involved in CF AECs, leading to this hyper-activation. Therefore, in this reports we sought to understand the differences between non-CF and CF airway epithelial cell activation of ERK1/ERK2 in response to PsaDM.

2. Materials and methods

2.1. Materials

Compound 1 (C1) was kindly provided by Professor Sir Philip Cohen (MRC PPU, University of Dundee, UK). PD153035 was bought from USBiological (Swampscott, MA, USA). *P. aeruginosa* diffusible material (PsaDM) was prepared and used as described previously [9].

Abbreviations: AEC, airway epithelial cells; CF, cystic fibrosis; CFTR, CF transmembrane regulator; EGF, epidermal growth factor; EGFR, EGF receptor; ERK, extracellular signal-regulated kinase; MAPK, mitogen activated protein kinase; PsaDM, *P. aeruginosa* diffusible material; TLR, toll-like receptors; TPL2, tumor progression locus 2.

* Corresponding author. Address: Meakins-Christie Laboratories, 3626 St-Urbain, Montréal H2X 2P2, Canada. Fax: +1 514 398 7483.
E-mail address: simon.rousseau@mcgill.ca (S. Rousseau).

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2.2. Antibodies

Anti-phospho Thr202/Tyr204 ERK1/ERK2 MAPK (4370) and Anti-ERK1/ERK2 MAPK (9107) were used at 1/1000 dilution and purchased from Cell Signaling (Danvers, MA, USA). Anti-phospho Tyr1068 EGFR (Ab40815) was purchased from Abcam (Cambridge, MA, USA) and used at 1/50 for immunochemistry. Goat anti-rabbit IgG DyLightTM800 (35571) and Goat anti-mouse IgG DyLightTM680 (35518) were used at 1/15000 dilution and were bought from Thermo Scientific (Rockford, IL, USA).

2.3. Cell lysis, RNA extraction, real-time PCR, immunoblotting and immunochemistry

All these techniques were performed as previously described [9].

2.4. Cell culture

Human airway epithelial cell line NuLi-1 and CuFi-1 (CFTRΔF508) were purchased from the ATCC (Rockville, MD, USA) and cultured as described previously [9]. Cells were grown to confluence and starved overnight before stimulation with PsaDM and/or inhibitors to prevent supplements-dependent ERK1/ERK2 and EGFR activation.

2.5. Statistical analysis

Analyses of variance (ANOVA) followed by a multiple comparison test (Bonferroni) were used to test differences in mean between groups (GraphPad Prism software, version 5.0). *p*-values less than 0.05 were considered to be significant.

3. Results and discussion

3.1. EGFR is phosphorylated in the CF airways

A recent report has found that inhibition of CFTR led to EGFR-dependent CXCL8 synthesis [10]. As we have previously reported that in CF AECs, the absence of CFTR led to higher MAPK activation and CXCL8 synthesis, we wondered whether the EGFR might play a role in ERK1/ERK2 MAPK activation and subsequent pro-inflammatory cytokine production. We first examined the phosphorylation of the EGFR at Tyr1068 by immunohistochemistry of non-CF and CF airway biopsies. We found a striking increase in the EGFR phosphorylation of the airway epithelium (black arrows) as well as sub-epithelial inflammatory cells (yellow arrows) in CF lung biopsies compared to non-CF biopsies (Fig. 1). This shows that EGFR is highly activated in CF inflamed airways. The presence of activated EGFR in inflammatory cells was unexpected but in accordance with reports showing expression on both neutrophils and monocytes [11,12].

3.2. TPL2 and EGFR contribute to ERK1/ERK2 hyper-activation in CF AECs

We have shown in the normal bronchial airway epithelial cell line BEAS-2B that TLR-mediated ERK1/ERK2 phosphorylation was dependent on the protein kinase TPL2 [8]. The presence of phosphorylated EGFR in CF AECs suggests that this receptor may also contribute to ERK1/ERK2 MAPK activation. In order to determine whether TPL2 and/or EGFR played a role in the hyper-activation of ERK1/ERK2 of CF AECs exposed to PsaDM, we prevented the activity of each protein kinase using pharmacological inhibition. TPL2 activity was prevented using Compound 1 (C1) as previously reported [8] and EGFR tyrosine kinase activity was blocked with PD153053 [13].

The non-CF AECs (NuLi) or CF AECs (CuFi) were pre-treated with C1 (2 μM) or PD153053 (100 nM) for 1 h before exposure for 15 min to PsaDM (5 μg/mL). These doses were selected because they were the lowest ones required to prevent ERK1/ERK2 phosphorylation in AECs [8]. As previously reported, ERK1/ERK2 activation was greater in the CF AECs (Fig. 2) [9]. Moreover, blocking TPL2 completely prevented ERK1/ERK2 phosphorylation in both CF and non-CF AECs (Fig. 2). Interestingly, blocking the EGFR also reduced ERK1/ERK2 activation, however this was markedly more pronounced in the CF AECs than in the non-CF AECs (70% inhibition in the CF AECs vs 35% inhibition in the non-CF AECs). The results highlight an interesting scenario, whereas TPL2 is essential for ERK1/ERK2 activation to PsaDM in both cell lines and the EGFR has a much greater contribution to ERK1/ERK2 activation in CF AECs. This means that the absence of CFTR modifies the cellular environment leading to a stronger EGFR activation following PsaDM challenge. This may explain the MAPK-hyper-activation we initially reported [9]. Although the mechanism linking CFTR to EGFR is not known, our results demonstrate that TPL2 is nevertheless required and may be found in a pathway upstream of the EGFR, something that has not been previously reported.

Interestingly, the contribution of the EGFR pathway may become even more important for prolonged exposure to infection as we have shown a greater and more sustained increase in the synthesis of the EGFR pro-ligand, Heparin-Binding EGF in CF AECs exposed to PsaDM (Fig. 3). These results imply that during a chronic infection a greater reservoir of EGFR ligands may be present and can explain the striking phosphorylation we have observed in CF lungs (Fig. 1).

3.3. CXCL8 synthesis is more dependent on the EGFR in CF AECs compared to non-CF AECs exposed to PsaDM

We showed that MAPK hyper-activation led to higher CXCL8 synthesis, likely due to an imbalance in antioxidant defence resulting in higher levels of reactive oxygen species [14], we therefore checked what was the contribution of TPL2 and EGFR to the synthesis of this pro-inflammatory mediator in CF AECs. Blocking TPL2 activity decreased the mRNA expression of CXCL8 by approximately half in both CF and non CF AECs (56% inhibition in the CF AECs vs 45% inhibition in the non-CF AECs) (Fig. 4). Blocking EGFR activity barely decreased CXCL8 mRNA expression in non-CF AECs (8%) whereas the decrease was much more pronounced in the CF AECs (24%), which fits with the greater contribution of EGFR to ERK1/ERK2 MAPK activation (Fig. 2). Naturally, other pathways, such as the NFκB and p38 MAPK are involved in controlling the synthesis of CXCL8 [14,15], explaining why the inhibition is not as strong for CXCL8 expression in the presence of the inhibitors as it is for ERK1/ERK2 activation. This is in accordance with the recent finding of Kim and colleagues, where they found that the absence of CFTR in the IB3 cell line or its inhibition led to EGFR-dependent CXCL8 synthesis [10].

Interestingly, although our data and that of Kim et al., support the view that EGFR activation is an aberrant signal resulting from the loss of CFTR, from a therapeutic stand point it may be worth considering targeting TPL2 instead of EGFR. The data in Fig. 4 shows that inhibition of TPL2 is more potent at decreasing CXCL8 levels and actually brought back the amount of CXCL8 to that of the normal AECs. This would suggest that you could remove excessive inflammation while keeping a response to infection. Moreover, activation of EGFR has been linked with a protective response to epithelial injury [16] and therefore blocking this receptor may have serious detrimental effects in the context of CF. Furthermore, the EGFR is linked to the activation of other pathways in addition to

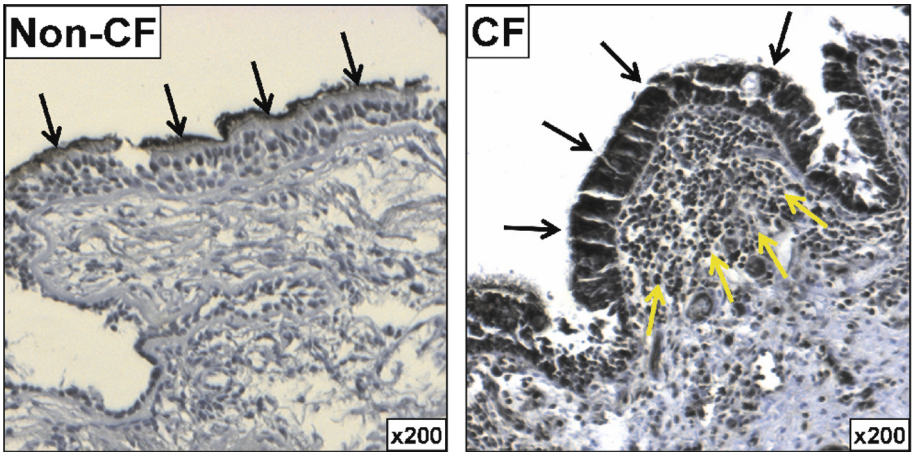

Fig. 1. EGFR activation in the airway epithelium of CF patients. Phosphorylated EGFR (phospho-Tyr1068) was detected by immunohistochemistry in tissue sections from CF and non-CF lungs (black arrows). Inflammatory cells were also stained in the CF biopsy (yellow arrows). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

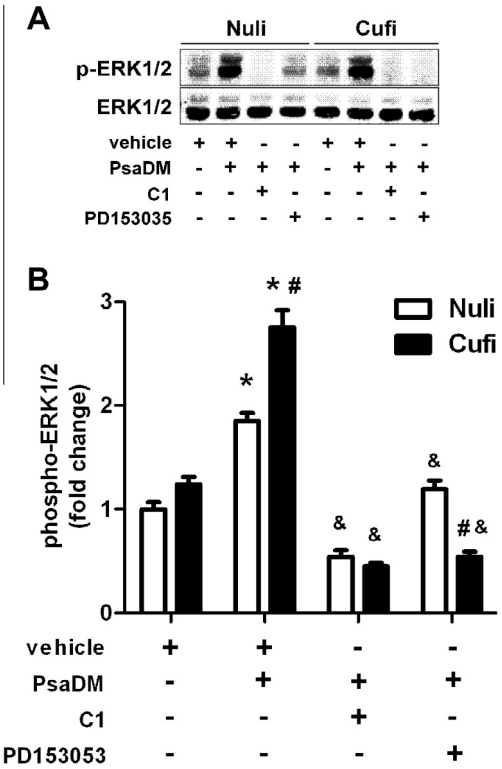

Fig. 2. AECs activation of ERK1/ERK2 is dependent on TPL2 and enhanced by EGFR in CFTRΔF508 cells during PsaDM stimulation. Non-CF (NuLi) or CFTRΔF508 (CuFi) AECs were pre-treated for 1 h with vehicle, 2 μM C1 or 100 nM PD153035 and stimulated with 5 μg/ml PsaDM for 15 min. Cells were lysed and the lysates subjected to SDS–PAGE. (A) Immunoblotting was performed with an antibody that recognizes ERK1/ERK2 phosphorylated at Thr202/Tyr204 (p-ERK1/2, upper panel) or an antibody that recognizes all forms of ERK1/ERK2 (ERK1/2, lower panel). (B) Quantitative analysis of the signal intensity obtained with an antibody recognizing only the phosphorylated forms of ERK1/ERK2 normalized to the signal obtained with antibody that recognizes all forms of ERK1/ERK2 was performed using Li-Cor infrared Odyssey imaging system. Indications of significance correspond to *p*-values associated to untreated cells (*), to PsaDM-treated cells (&) or to the corresponding NuLi treated-cells (#). Representative blots from four distinct experiments are shown. Quantitative analysis of the signals was performed and expressed as graphs.

the ERK1/ERK2 MAPK, therefore its inhibition may lead to potentially more unwanted effects than that of the protein kinase TPL2, which so far has a much narrower range of target. Naturally,

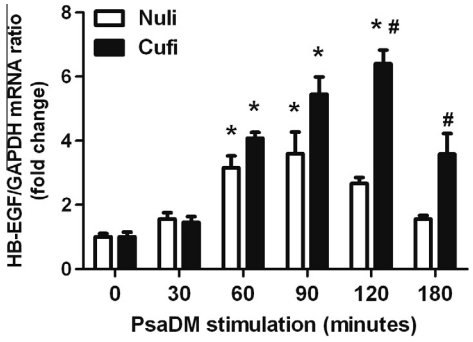

Fig. 3. PsaDM stimulation upregulates EGFR pro-ligand expressions and is enhanced in CFTRΔF508 cells. Non-CF (NuLi) or CFTRΔF508 (CuFi) AECs were left untreated or stimulated with 5 μg/ml PsaDM for the indicated time. Total RNA was extracted and subjected to QRT-PCR for HB-EGF. Indications of significance correspond to *p*-values associated to untreated cells (*), to PsaDM-treated cells (&) or to the corresponding NuLi treated-cells (#). Results from four independent experiments are shown.

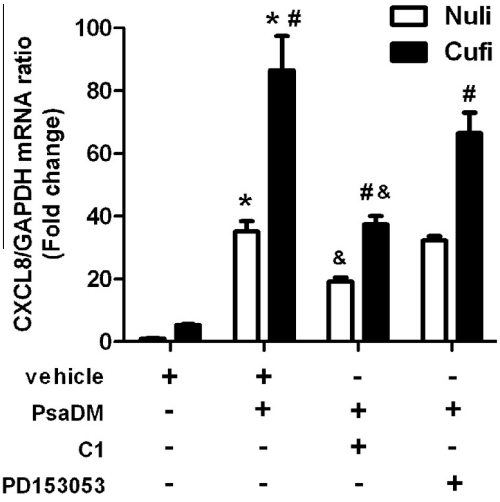

Fig. 4. EGFR-dependent CXCL8 expression is exclusive to CFTRΔF508 cells. Non-CF (NuLi) or CFTRΔF508 (CuFi) AECs were left untreated or pre-treated for 1 h with vehicle, 2 μM C1 or 100 nM PD153035 and stimulated with 5 μg/mL PsaDM for 1 h. Total RNA was extracted and subjected to QRT-PCR for CXCL8. Indications of significance correspond to *p*-values associated to untreated cells (*), to PsaDM-treated cells (&) or to the corresponding Nuli treated-cells (#). Results from four independent experiments are shown.

these are speculations based on *in vitro* data and should be demonstrated in *in vivo* models.

In conclusion, in this report we have defined a novel network leading to ERK1/ERK2 activation in CF AECs in response to *P. aeruginosa* that involves both the TPL2 and EGFR protein kinases and contributes to inflammation of CF airways.

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