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Mechanisms of endothelial cell dysfunction in cystic fibrosis

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ABSTRACT

Although cystic fibrosis (CF) patients exhibit signs of endothelial perturbation, the functions of the cystic fibrosis conductance regulator (CFTR) in vascular endothelial cells (EC) are poorly defined. We sought to uncover biological activities of endothelial CFTR, relevant for vascular homeostasis and inflammation. We examined cells from human umbilical cords (HUVEC) and pulmonary artery isolated from non-cystic fibrosis (PAEC) and CF human lungs (CF-PAEC), under static conditions or physiological shear. CFTR activity, clearly detected in HUVEC and PAEC, was markedly reduced in CF-PAEC. CFTR blockade increased endothelial permeability to macromolecules and reduced trans-endothelial electrical resistance (TEER). Consistent with this, CF-PAEC displayed lower TEER compared to PAEC. Under shear, CFTR blockade reduced VE-cadherin and p120 catenin membrane expression and triggered the formation of paxillin- and vinculin-enriched membrane blebs that evolved in shrinking of the cell body and disruption of cell-cell contacts. These changes were accompanied by enhanced release of microvesicles, which displayed reduced capability to stimulate proliferation in recipient EC. CFTR blockade also suppressed insulin-induced NO generation by EC, likely by inhibiting eNOS and AKT phosphorylation, whereas it enhanced IL-8 release. Remarkably, phosphodiesterase inhibitors in combination with a β_2 adrenergic receptor agonist corrected functional and morphological changes triggered by CFTR dysfunction in EC. Our results uncover regulatory functions of CFTR in EC, suggesting a physiological role of CFTR in the maintenance EC homeostasis and its involvement in pathogenetic aspects of CF. Moreover, our findings open avenues for novel pharmacology to control endothelial dysfunction and its consequences in CF.

1. Introduction

Cystic fibrosis (CF) is a genetic disease, caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene [1]. The CFTR protein regulates Cl^- and $HCO3^-$ secretion in ciliated and serous cells of submucosal glands and ducts, and in epithelial cells [2,3]. In the airways, CFTR dysfunction reduces the periciliary fluid volume and impairs the mucociliary clearance, promoting infection, inflammation and respiratory insufficiency [4]. CFTR dysfunction in cells deputed to the immune-surveillance and host response can contribute to CF pathogenesis [5–7].

The vascular endothelium is pivotal for the regulation of vascular

Abbreviations: AJ, adherent junctions; CFTR, cystic fibrosis conductance regulator; ECGF, endothelial cell growth factor; EMV, endothelial microvesicles; eNOS, endothelial nitric oxide synthase; HUVEC, human umbilical vein endothelial cells; PAEC, pulmonary artery endothelial cells; PDE, phosphodiesterase; PKA, protein kinase A; TEER, trans-endothelial electrical resistance; TNF-α, tumor necrosis factor-α; YFP, yellow fluorescent protein

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homeostasis and of the inflammatory response. It forms a semi-permeable barrier between tissues and the bloodstream, thereby regulating solute transport and immune cells trafficking. The endothelial barrier function is tightly controlled by intercellular adherence junctions (AJ) and tight junctions, interconnected with cytoskeletal proteins [8]. The transmembrane protein Vascular Endothelial cadherin (VE-cadherin) is the major structural component of endothelial AJ. p120-catenin protects VE-cadherin from being internalized and is required for the proper assembly of AJ and the maintenance of barrier function [9]. Endothelial cells (EC) are also engaged in transcellular exchanges by releasing either soluble mediators, including nitric oxide (NO) and interleukin (IL)-8 that control vascular tone and leukocyte recruitment, or microvesicles (EMV) that carry a repertoire of proteins, lipids and nucleic acids [10].

Despite its preeminent role in vasoregulation and inflammation, the vascular endothelium has been insufficiently investigated in CF. CFTR expression in EC and the involvement of CFTR in endothelial responses to hypoxia have been reported [11,12]. We and others documented that CF patients present peripheral biochemical signs of endothelial perturbation [13,14]. Moreover, microvascular dysfunction in CF patients has been recently reported [15]. Together with enhanced oxidant stress, unresolved inflammation and the extension of life expectancy, endothelial dysfunction could contribute to increase the cardiovascular risk for CF patients.

In the present report, we investigated the impact of CFTR loss-of-function on EC activities potentially related to cardiovascular risk and lung inflammation.

2. Materials and methods

DMEM and 199 media, fetal calf serum (FCS), penicillin and streptomycin were from Gibco (Waltham, Massachusetts, U.S.A.). HEPES was from Merk (Darmstadt, Germany). CFTRinh-172, formoterol fumarate and anti-p120 catenin antibody were from Santa Cruz Biotechnology (Dallas, Texas, U.S.A.). IL-1β (human, recombinant) was from Enzo Life Sciences (NY, USA). Roflumilast N-Oxide (RNO), the active metabolite of roflumilast was generously provided by Nycomed (Konstanz, Germany). Rolipram, Formoterol and Cilostamide were from Calbiochem (Vimodrone, Milano, Italy). Anti-VE-Cadherin antibody and DRAQ5 were from Abcam (Cambridge, U.K.). Anti-paxillin and anti-vinculin antibodies were from BD (BD Trasduction Laboratories, Milano Italy) and Sigma-Aldrich (St Louis, MO, USA), respectively. Phalloidin-TRITC and fluorescein isothiocyanate (FITC)-conjugated dextran (FD4; 3-5kDA) were from Sigma (Saint Louis, MO, U.S.A.).

2.1. Cells

Human umbilical vein endothelial cells (HUVEC) were isolated as previously described [16] and cultured in DMEM/199 medium (50% volume), endothelial cell growth supplement (100 μg/mL), heparin (15 U/mL) penicillin-streptomycin (100 μg/mL, each) and FCS (12%) at 37 °C under 5% CO_2. Experiments were performed using cells up to the third passage.

Human pulmonary artery endothelial cells (PAEC) were isolated from surgical fragments. Segments of human arterial pulmonary endothelial cells (PAEC) were rinsed three times with a solution made of 3.9% saline containing 1% penicillin/streptomycin and fungizone (0.25 mg/mL) in order to remove blood cells and other contaminants. Specimens were then washed three times with 3.9% saline containing 250 μg/mL linezolid, 50 U/mL colistin, 25 μg/mL co-trimoxazole and 25 μg/mL amphotericin B and thereafter placed in 50 mL tubes containing DMEM/M199 (50%/50%), 10% FBS, 1% L-Glutamine (200 mmol/L), 1% Penicillin/Streptomicin (100 ×), 1% Fungizone (0.25 mg/mL), 1% ECGF (100 ×), 1% heparin (100 ×) plus 250 μg/mL linezolid, 50 U/mL colistin, 25 μg/mL co-trimoxazole and 25 μg/mL amphotericin B. Samples were maintained at 4 °C until exposed (usually within 6–12 h from surgical excision) for 20 min at 37 °C, 5% CO_2, 90% humidity to phosphate-buffered saline (PBS) without calcium and magnesium containing 2 mg/mL type II collagenase (Worthington Biochem, Lakewood, NJ). Immediately after the incubation, samples were moved into a Petri dish (100 mm) containing culture medium (DMEM-M199, 50% vol/vol), supplemented with 10% FBS, 1% L-Glutamine, 1% Penicillin/Streptomicin, 1% Fungizone, 1% ECGF, 1% heparin (EC medium), together with 250 μg/mL linezolid, 50 U/mL colistin, 25 μg/mL co-trimoxazole and 25 μg/mL amphotericin B, which were progressively reduced and removed after a week from the explant. The artery fragments were massaged with a spatula followed by gentle shacking to detach the endothelial cells, which were collected by centrifugation at 330 x g for 7 min at room temperature. The cell pellet was suspended with EC medium and seeded on fibronectin (1 mg/cm^2) (Sigma-Aldrich, Saint Louis, MO, USA)-coated cell culture dishes. Cells were maintained at 37 °C, 5% CO_2, 90% until confluent. With this method, the percentage of cells expressing the endothelial phenotype was variable from 10 to 50%.

To obtain a virtually pure endothelial population, we enriched EC using a method developed in our laboratory, based on early adhesion of EC compared to contaminant cells [17], which gave 95–98% endothelial cells after 3–4 passages. The endothelial phenotype was assessed by immunofluorescence (Suppl. Fig. 1). To this end, 2×10^4 cells were seeded on glass coverslips pre-coated with 1.5% gelatin. The next day cells were washed with PBS, fixed with 4% paraformaldehyde, permeabilized with 0.5% saponin and stained overnight at 4 °C with anti VWF (MA5-14029 Pierce) and CD31 (M0823 Dako) antibodies. Cells were subsequently exposed to Alexa Fluor® 488-labeled goat anti-Mouse IgG for 1 h at 4 °C. Draq-5 was used for nuclei staining. High definition images were acquired using a Zeiss LSM-510 META system (Carl Zeiss, Jena, Germany), equipped with a Zeiss Axiovert 200 inverted microscope.

2.2. CFTR expression

2.2.1. Western blotting

Cells were harvested and scraped into lysis buffer (50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1% Triton, 1% deoxycholate, 0.1% SDS) supplemented with protease inhibitors (Roche, Indianapolis, IN, USA). Lysates were incubated in a rocking platform for 30 min at 4 °C and then centrifuged at 13,000 rpm for 30 min at 4 °C to remove debris. Proteins were quantitated with a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA), separated by SDS-PAGE and transferred on nitrocellulose membrane. After exposure to 5% nonfat milk in PBS for 1 h, membranes were probed with a specific primary antibody: anti-CFTR mAb clones L12B4 and M3A7 (1:100 each; Merck Millipore, Darmstadt, Germany). Anti-α-tubulin mAb (1:4000; Sigma-Aldrich, St. Louis, MO, USA) was used as loading control. The binding of primary antibodies was detected with anti-mouse secondary peroxidase-conjugated antibody (1:1000 to detect CFTR, 1:7500 for α-tubulin; Merck Millipore), and visualized with the clarity western ECL substrate (Bio-Rad, Hercules, CA, USA). Immunoblot images were acquired with Alliance 4.7 (UVITEC, Cambridge, UK). The intensity of the relevant bands was evaluated using the UVI band software package or with the ImageJ NIH Image Analysis Program.

2.2.2. Flow cytometry

CFTR expression in non-CF and CF-PAEC was also evaluated by flow cytometry. For surface localization, cells were detached with EDTA and collected by centrifugation (300 × *g* for 10 min). Pellets were suspended with PBS and incubated (1 h at 4 °C) with a mouse monoclonal anti-human CFTR antibody (CF3) (ThermoFisher) directed against the amino acid residues 103–107 located within the first extracellular loop. After removing the excess of primary antibody by centrifugation wash, cells were incubated with PerCP/Cy5.5-conjugated anti-mouse IgM (BioLegend, San Diego, CA, USA) for 1 h at 4 °C. For evaluation of