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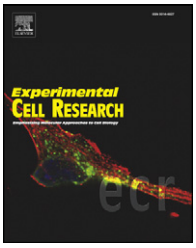

Research Article

In vivo inhibition of epidermal growth factor receptor autophosphorylation prevents receptor internalization

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ABSTRACT

The question whether epidermal growth factor (EGF)-induced receptor endocytosis requires the prior autophosphorylation via the EGF receptor (EGFR) kinase domain has been a matter of long-standing debate. In the airway epithelial cell line NCI-H292, the EGFR kinase domain inhibitor BIBW 2948 BS was found to inhibit both autophosphorylation and subsequent internalization of the endogenous EGFR with similar IC_{50} values. Applying an *ex vivo* EGFR internalization assay in a clinical study, the *in vivo* effect of inhalatively administered BIBW 2948 BS was determined directly at the targeted receptor in airway tissues from COPD patients. In these experiments, the *in vivo* inhibition of the EGFR kinase domain prevented the EGF-induced internalization of EGFR.

Introduction

Epidermal growth factor receptor (EGFR) expression has been shown to be up-regulated in chronic hypersecretory airway diseases such as asthma, chronic bronchitis, and chronic obstructive pulmonary disease (COPD) [1]. Oxidative stress associated with cigarette smoke is known to activate EGFR [2]. Activated EGFR in turn induces airway goblet cell hyperplasia and mucin production from these goblet cells; both effects could be prevented by treatment with specific EGFR tyrosine kinase (TK) inhibitors [2–4].

The question whether the auto-/transphosphorylation of the EGFR kinase domains is a prerequisite for EGF-induced receptor endocytosis has been contentious for more than two decades [5–11]. One reason for the partially contradictory experimental results may be the use of different model cell lines, sometimes recombinantly overexpressing the EGFR to non-physiologically high levels. With the endogenous expression levels of EGFR in NCI-H292 airway epithelial cells, we demonstrate that both BIBW 2948 BS and AG1478, specific inhibitors of the EGFR TK activation, suppressed both EGFR autophosphorylation and internalization with virtually identical IC_{50} values. Secondly, we found that BIBW 2948 BS, when inhalatively administered to COPD patients in the course of a double-blind clinical phase II study [12], prevented the subsequent EGF-stimulated EGFR internalization in an *ex vivo* assay using airway biopsies.

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Methods

EGFR internalization experiments in NCI-H292 cells

EGFR antibody co-internalization

NCI-H292 (ATCC®, #CRL-1848) cells were cultured on 384 well imaging-suitable collagen I-coated microtiter plates (Falcon BD) in an RPMI 1640 medium with L-Glutamine (BioWhittaker, #BE12-702F), 1% Pen/Strep (Biochrom, #A2213), and 10% fetal bovine serum (FBS; Gibco, #10500-064), hereafter referred to as the "culture medium", and incubated at 37 °C, 5% CO_2 overnight. EGFR antibody co-internalization experiments were performed using an FITC-conjugated antibody directed against an extracellular epitope of human EGFR at a dilution 1:500 (mouse monoclonal IgG-type antibody; Biotrend, #4222-0237). The EGFR antibody was incubated with NCI-H292 cells for 30 min at 37 °C, 5% CO_2. The cells were stimulated with the indicated concentration of recombinant human EGF (Sigma-Aldrich, #E9644) for 30 min at 37 °C/5% CO_2, then fixed for 30 min at room temperature (RT) with 4% formaldehyde (FA)/phosphate-buffered saline (PBS) solution (Sigma-Aldrich, #25,254-9). In parallel to the fixation process, the cell nuclei were stained by adding 1 μM Hoechst 33342™ dye (Molecular Probes, #H-3570) to the fixation medium.

AF647™-EGF internalization

The NCI-H292 cells were cultured as described above. EGFR detection with fluorescently labeled EGF was achieved by incubating the cells with the indicated concentrations of AF647™-coupled EGF (Molecular Probes, #E-35351) for 30 min at 37 °C/5% CO_2 and fixed as explained in the section EGFR antibody co-internalization.

EGFR phosphorylation experiments

To detect activated EGFR, cells were stimulated with unlabeled EGF (5 min, 37 °C, 5% CO_2), fixed and treated for 30 min at RT with 0.05% saponin/1% BSA. NCI-H292 cells were incubated either with an EGFR pTyr1086-specific antibody (dilution 1:5000, rabbit monoclonal antibody; Epitomics, #1139-1) or with an antibody recognizing tyrosine phosphorylated EGFR (dilution 1:300, mouse polyclonal antibody; BD Transduction Laboratories, #610025). Primary antibodies were detected with respectively goat–anti-rabbit or goat–anti-mouse AF647™-conjugated IgG antibodies at a dilution 1:1000 (Molecular Probes, #A-21245 and #A-21236, resp.).

EGFR kinase domain inhibition

EGFR kinase domain inhibitors BIBW 2948 BS and AG1478 were from Boehringer Ingelheim Pharma GmbH & Co KG and from Tocris Bioscience (#1276), resp. Prior to EGFR internalization or phosphorylation experiments, NCI-H292 cells were incubated with BIBW 2948 BS or AG1478 in the culture medium containing 1% DMSO for 30 min at 37 °C/5% CO_2.

Subjects and design of the clinical study

The clinical trial was registered at ClinicalTrials.gov (Identifier NCT00423137). The study was a randomized, double-blind, placebo-controlled, parallel group study to evaluate the effects of 4-week treatment of BIBW 2948 BS on epithelial mucin stores in COPD patients with symptoms associated with chronic bronchitis. Male and female active smokers aged between 40 and 70 years with a history of COPD and chronic bronchitis were eligible. Baseline post-bronchodilator forced expiratory volume in the first second (FEV1) had to be equal or greater than 40% of what is predicted and the FEV1 had to be less than 70% of the forced vital capacity. In total, 101 patients consented to the study; from 25 patients bronchial brushings were available in duplicate (at baseline and at end-of-treatment). In one patient, samples were not suitable for further object segmentation and image analysis due to weak nucleus staining in the baseline sample. The study was performed at 6 academic sites in the USA and Germany and had been approved by all concerned institutional review boards.

Analysis of EGFR internalization in human primary airway epithelial cells from patients included in the clinical study

EGFR internalization experiments were performed with human primary airway epithelial cells acquired during two subsequent bronchoscopies (baseline and end-of-treatment). Cells were obtained from two separate airway epithelial brushings from lower-lobe segments using disposable cytology brushes. The cells were resuspended in 400 μl complete phenol red-free DMEM (Gibco, #21063) containing 10% FCS by careful stirring on a Vortex. Five 80 μl aliquots of the cell suspension were transferred to five wells of a collagen I-coated microtiter plate (96 well View Plate; Perkin Elmer). The plates were subsequently incubated (30 min, 37 °C, 5% CO_2), so that the cells could attach to the bottom of the microtiter plate wells. 20 μl of 500 ng/ml AF647™-EGF in a complete medium was added to four of the five wells, the remaining well received 20 μl of the complete medium (background control). The microtiter plate was incubated for 30 min (37 °C, 5% CO_2). EGFR internalization was stopped with 4% FA/1 μM Hoechst 33342™ dye and supplemented with DMSO to a final concentration of 10%. Cells from patients were then frozen on dry ice, shipped to the HCA unit at Boehringer Ingelheim Pharma GmbH & Co. KG (Biberach) and thawed at RT before imaging of the samples was performed. 30 different images per well were analyzed with regard to EGFR internalization using Definiens Enterprise Image Intelligence™ Software (see below).

IN Cell Analyzer 3000™

All images of the respective experiments were taken using the IN Cell Analyzer 3000™ (General Electric [GE] Healthcare). For all experiments, the 364 nm laser line combined with the 450BP65 emission filter was employed for the Hoechst 33342™ dye. For EGFR antibody co-internalization experiments, we employed the 488 nm laser line combined with the 535BP45 emission filter for FITC; fluorescence emission was sequentially recorded in the green and in the blue channel. To record the images of internalized AF647™-EGF and pEGFR-directed antibodies, the 647 nm laser line was employed combined with the 695BP55 emission filter.

Image analysis of EGFR internalization and phosphorylation in NCI-H292 cells

Internalized EGFR in NCI-H292 cells was quantified using the GRN2™ module and pEGFR was quantified using the RAY0™ module; both are part of the IN Cell Analyzer 3000™ image analysis software.