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Cigarette smoke extract induced exosome release is mediated by depletion of exofacial thiols and can be inhibited by thiol-antioxidants

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ABSTRACT

Introduction: Airway epithelial cells have been described to release extracellular vesicles (EVs) with pathological properties when exposed to cigarette smoke extract (CSE). As CSE causes oxidative stress, we investigated whether its oxidative components are responsible for inducing EV release and whether this could be prevented using the thiol antioxidants N-acetyl-L-cysteine (NAC) or glutathione (GSH).

Methods: BEAS-2B cells were exposed for 24 h to CSE, H_2O_2, acrolein, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), bacitracin, rutin or the anti-protein disulfide isomerase (PDI) antibody clone RL90; with or without NAC or GSH. EVs in media were measured using $CD63^+CD81^+$ bead-coupled flow cytometry or tunable resistive pulse sensing (TRPS). For characterization by Western Blotting, cryo-transmission electron microscopy and TRPS, EVs were isolated using ultracentrifugation. Glutathione disulfide and GSH in cells were assessed by a GSH reductase cycling assay, and exofacial thiols using Flow cytometry.

Results: CSE augmented the release of the EV subtype exosomes, which could be prevented by scavenging thiol-reactive components using NAC or GSH. Among thiol-reactive CSE components, H_2O_2 had no effect on exosome release, whereas acrolein imitated the NAC-reversible exosome induction. The exosome induction by CSE and acrolein was paralleled by depletion of cell surface thiols. Membrane impermeable thiol blocking agents, but not specific inhibitors of the exofacially located thiol-dependent enzyme PDI, stimulated exosome release.

Summary/conclusion: Thiol-reactive compounds like acrolein account for CSE-induced exosome release by reacting with cell surface thiols. As acrolein is produced endogenously during inflammation, it may influence exosome release not only in smokers, but also in ex-smokers with chronic obstructive pulmonary disease. NAC and GSH prevent acrolein- and CSE-induced exosome release, which may contribute to the clinical benefits of NAC treatment.

1. Introduction

Situated at the vast interface between environment and lung tissue, the airway epithelium is crucial for maintaining pulmonary homeostasis. Not only does it act as a physical barrier, it also supports appropriate responses to inhaled pathogens and chemicals by sending signals towards immune and stromal cells in its vicinity (for a review, see [1]). Yet, epithelial integrity and function become impaired upon

Abbreviations: AEC, airway epithelial cells; AFM, Alexa Fluor 488 Maleimide; BSA, bovine serum albumin; COPD, chronic obstructive pulmonary disease; CSEt, cigarette smoke extrac; DMSO, dimethylsulfoxide; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; EVs, extracellular vesicles; FACS, fetal calf serum; GSH, glutathione; GSSG, glutathione disulfide; H_2O_2, hydrogen peroxide; MTT, thiazolyl blue tetrazolium bromide; NAC, N-acetyl-L-cysteine; PBS, phosphate buffered saline; PDI, protein disulfide isomerase; PE, phycoerythrin; PI, propidium iodide; RFU, relative fluorescence units; ROS, reactive oxygen species; TRPS, tunable resistive pulse sensing

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chronic exposure to noxious stimuli, for instance in long term cigarette smokers [2]. Cigarette smoke-induced epithelial damage is partly mediated by components that cause oxidative stress, such as the reactive oxygen species (ROS) hydrogen peroxide (H_2O_2) [3,4] and the reactive carbonyl acrolein [5,6]. The oxidative stress exerted by cigarette smoke components is thought to contribute to the early pathogenesis of chronic obstructive pulmonary disease (COPD) [7]. Moreover, once COPD is established, markers of oxidative stress, as well as acrolein, remain elevated in the lungs even after patients stop smoking [8,9]. Recent studies have revealed that treatment with a high-dose of the thiol-group bearing mucolytic and antioxidant N-acetyl-L-cysteine (NAC) improved small airway function in COPD patients [10] and lowered the frequency of acute exacerbations [11]. This suggests that oxidative stress causally contributes to small airway disease and worsening of COPD *via* acute exacerbations, even in ex-smokers.

A number of studies have suggested that oxidative stress influences the release of extracellular vesicles (EVs) by various cell types [12–14]. EVs are membrane vesicles secreted by nearly all cell types. They consist of a lipid bilayer surrounding an aqueous lumen, and act as carriers for molecules derived from their cell of origin, including membrane proteins, cytoplasmic proteins and RNA. EV functions include removing unnecessary or toxic molecules from their cell of origin [15,16], as well as delivering complex messages between cells [17]. Thus, EVs regulate many biological processes, including immune responses and inflammation [17]. Although EV nomenclature is complex and still controversial [18], EVs are commonly classified into two major groups based on their size and marker expression, the larger microvesicles and the smaller exosomes. In the context of cigarette smoke-induced lung disease, microvesicles have been studied predominantly to evaluate their utility as biomarkers for early lung damage and exacerbation susceptibility [19,20]. In contrast, the smaller exosomes have been investigated for functional properties that may contribute to COPD pathogenesis. Two recent *in vitro* studies have suggested that exosomes secreted by airway epithelial cells (AEC) exposed to cigarette smoke extract (CSE) cause inflammation [21] and tissue remodeling by inhibiting fibroblast autophagy [22]. Yet, it has not been clarified how CSE influences the exosome release by AEC and whether this can be manipulated. We hypothesized that oxidative CSE components, such as H_2O_2 or acrolein, mediate CSE-induced exosome release and that the exosome induction can be prevented by antioxidants.

2. Materials and methods

2.1. Cell culture

BEAS-2B human bronchial epithelial cells (ATCC CRL-9609) were cultured at 5% CO_2 and 37°C in T-75 culture flasks pre-coated with LHC basal medium (Gibco, Life Technologies, New York, NY, USA) supplemented with 0.1 mg/ml bovine serum albumin (BSA, Boehringer Mannheim GmbH, Mannheim, Germany), 0.03 mg/ml bovine collagen I (BD Biosciences, San Jose, CA, USA) and 0.01 mg/ml human fibronectin (BD Biosciences). Cells were subcultured twice per week in RPMI1640 (Gibco) containing 10% fetal calf serum (FCS, Lonza, Verviers, Belgium).

2.2. EV-depletion of FCS

To deplete confounding bovine EVs, FCS was diluted to 30% (v/v) in DMEM-F12 without phenol-red (Gibco) and centrifuged for 16 h at 40,000 rpm (Average RCF = 117,734 × *g*), in a fixed-angle Type 70Ti-rotor in an Optima L-90K preparative ultracentrifuge (Beckman-Coulter, Brea, CA, USA). The supernatant was collected without disturbing the concentrated layer at the bottom of the tube.

2.3. Stimuli and cell exposures

N-acetyl-L-cysteine (NAC), H_2O_2, acrolein, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), bacitracin and rutin were from Sigma Aldrich (St. Louis, MO, USA). The monoclonal anti-protein disulfide isomerase (PDI) antibody clone RL90 was from Thermo Fisher Scientific (Waltham, MA, USA). To produce CSE, mainstream smoke of one cigarette (Reference Cigarette 3R4F, Tobacco-Health Research, University of Kentucky, USA) without filter was drawn through 2 ml phosphate buffered saline (PBS) using a vacuum-pump at constant speed. The obtained solution was filtered through a 0.22 µm Acrodisk filter (Pall, Port Washington, NY, USA) and defined as 100% CSE. For quality control, the extinction of 100 µl 100% CSE was measured in duplicate at wavelengths of λ = 320 nm (A320) and λ = 540 nm (A540). CSE quality was accepted if ΔOD (A320-A540) was between 0.9 and 1.2. CSE was prepared freshly for each experiment and used within 15 min.

BEAS-2B cells were seeded in RPMI1640 + 10% (v/v) FCS on pre-coated plates (5×10^4 cells/cm^2) and allowed to attach for 24 h. Cells were washed twice with PBS and incubated with reduction medium (DMEM-F12 + 0.1% (v/v) EV-depleted FCS, 0.25 ml/cm^2) for 2 h. Next, cells were exposed to different concentrations of CSE, acrolein, H_2O_2, DTNB, bacitracin, rutin or RL90 in reduction medium for 24 h. Where indicated, cells were treated with NAC (1 mM) or GSH (62.5, 125 or 250 µM) for 2 h. After these 2 h, cells were washed with PBS to remove any pre-formed EVs. Then, NAC or GSH were added again to the cells in combination with either CSE, acrolein or H_2O_2 and co-incubated for 24 h. Rutin was dissolved in DMSO, all other chemicals were dissolved and diluted in PBS. Vehicle controls were exposed to matched concentrations of DMSO or PBS. The vehicle control for RL90 contained 0.0005% (v/v) sodium azide.

2.4. MTT assay

Cells were washed with PBS and 200 µl/cm^2 reduction medium containing 0.5 mg/ml thiazolyl blue tetrazolium bromide (MTT; Sigma Aldrich) was added. After 3 h, medium was discarded and the formed formazan crystals were dissolved in 100 µl/cm^2 dimethyl sulfoxide (DMSO; Sigma Aldrich). Absorbance of the dissolved formazan was measured at λ = 540 nm and cell viability was expressed relative to a non-treated cells (100% cell viability) and triton-lysed cells (0% viability).

2.5. GSH measurements

For determining GSH oxidation in cell-free conditions, different CSE dilutions were made in PBS containing 50 µM reduced glutathione (Sigma Aldrich) and incubated for 24 h at 37°C. Ten microliter of each solution was transferred to a 96-well plate in triplicate. Ninety microliter of DTNB working reagent (1 mM DTNB and 2.5 mM sodium acetate in 0.1 M Tris-hydrochloride, pH 8.8) was then added. Absorbance was measured with a plate reader at λ = 412 nm.

For analyzing cellular GSH concentrations, 2×10^5 cells were washed twice with ice cold PBS. They were then incubated on ice with 250 µl 0.1 M potassium phosphate buffer containing 5 mM ethylenediaminetetraacetic acid (EDTA) disodium salt and 0.1% (v/v) Triton X-100, pH = 7.5 for 30 min. Next, cells were scraped and cellular debris was pelleted at 16,100 × *g* at 4 °C for 15 min. Supernatant was transferred to a new tube. Protein concentrations were determined using the Bradford assay according to the manufacturer's protocol (Bio-Rad, Hercules, CA, USA). The remaining supernatant was mixed 1:1 with 6% (m/v) sulfosalicylic acid. The samples were stored at −80 °C until analysis of GSH and the GSH oxidation product glutathione disulfide (GSSG) using an enzymatic cycling assay as previously described [23]. GSSG and GSH concentrations were normalized to the protein concentrations.