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Pro-inflammatory effects of extracellular Hsp70 and cigarette smoke in primary airway epithelial cells from COPD patients

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ABSTRACT

Extracellular Hsp70 (eHsp70) can activate immune cells via Toll-like receptors (TLR) 2 and 4, and induce cytokine synthesis. The aim of this study was to explore inflammation-associated effects of eHsp70 alone and in combination with cigarette smoke extract (CSE) in primary bronchial epithelial cells.

We assessed IL-6 and IL-8 concentrations, TLR2, TLR4 and Hsp70 mRNA expressions, and mitogen-activated protein kinases (MAPKs) activation induced by recombinant human (rh) Hsp70, CSE or their combinations in normal human bronchial epithelial cells (NHBE) obtained commercially, and primary bronchial epithelial cells isolated from non-COPD lung donors (PBEC) or COPD patients (PBEC COPD).

Baseline levels of IL-6 and IL-8 were significantly higher in PBEC COPD than in non-COPD PBECs. Upon rhHsp70 stimulation, IL-6 and IL-8 were significantly increased, with the strongest response in COPD-derived PBECs. CSE alone elevated cytokine secretion in all examined cells. rhHsp70 and CSE had antagonistic interactions on IL-8 release in PBECs from COPD patients, while the addition of rhHsp70 further increased CSE-induced IL-6 secretion in NHBE cells. rhHsp70 and CSE alone decreased TLR2 and TLR4 mRNA expression in COPD-derived PBECs. In non-COPD PBECs, combined treatments decreased only TLR2 mRNA expression. Hsp70 mRNA expression, as indicator of intracellular Hsp70, which may have anti-inflammatory effects, was reduced in COPD-derived cells upon exposure to CSE and rhHsp70 alone, but not with their combinations. Contrary to this, in PBECs from lung donors only combined treatments suppressed Hsp70 gene expression. CSE activated JNK and p38 MAPKs, while rhHsp70 increased activation of c-Jun kinase in NHBE cells.

Collectively, both eHsp70 and CSE induce pro-inflammatory responses in PBECs from non-COPD as well as COPD donors, but in combination antagonistic effects were observed in COPD-derived cells. These effects may be related to the regulation of TLR2/4 and might lead to modulation of inflammation with possible deleterious consequences for COPD patients.

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1. Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by chronic and progressive airflow limitation associated with an enhanced inflammatory response in the airways and the lung to noxious particles or gases, especially cigarette smoke [1]. It is a major cause of morbidity, mortality, and disability, especially in smokers, as around 80–90% of COPD patients are smokers [2]. Abnormal inflammatory responses to smoking in patients with COPD could develop due to the combination of genetic susceptibility and chronic exposure to cigarette smoke, but precise
molecular mechanisms of those processes are still unknown [3,4].

Extracellular Hsp70 (eHsp70) can act as damage-associated molecular pattern (DAMP) and induce inflammation in various cells and tissues. Those effects are triggered via Toll-like receptors (TLR) 2 and 4, leading to activation of nuclear factor xB (NF-kB) signal transduction pathway as well as mitogen-activated protein kinase (MAPK) pathways, and resulting in cytokine production [5–9].

The role of Hsp70 in COPD has not been elucidated in detail, but intracellular Hsp70 has been explored better than eHsp70. It was shown that cigarette smoke induces the synthesis of Hsp70 protein in monocytes, fibroblasts and 16HBE bronchial epithelial cells cultured in vitro [10–12]. In addition, elevated expression of Hsp70 that correlates with severity of the disease was found in lung epithelial cells of patients with COPD [11]. Contrary to this, in total leukocytes, lymphocytes, and alveolar macrophages of COPD patients the expression of Hsp70 was reduced compared to healthy controls and also depended on the smoking status of COPD patients [13–15].

Only a few studies so far measured the levels of extracellular Hsp70 in patients with COPD. Hacker et al. found higher concentrations of Hsp70 in the sera of COPD patients compared to healthy non-smokers [9]. Later, Hacker et al. [5] found that cigarette smoke induced Hsp70 in patients with COPD. Hacker et al. found higher concentration of Hsp70 in the sera of COPD patients compared to healthy non-smokers [9].

The aim of this study was to explore inflammation-associated effects of eHsp70 alone and in combination with cigarette smoke extract (CSE) by using three types of primary airway epithelial cells, namely normal human bronchial epithelial cells (NHBE) obtained commercially, primary bronchial epithelial cells isolated from non-COPD lung donors (PBEC) and primary bronchial epithelial cells isolated from COPD patients (PBEC COPD). We hypothesized that eHsp70 will alter CSE-induced responses, leading to more pronounced inflammatory effects. Therefore, we assessed concentrations of pro-inflammatory cytokines interleukin (IL)-6 and IL-8 after treatment with recombinant human (rh) Hsp70, CSE or their combinations. We also detected expression of TLR2, TLR4 and intracellular Hsp70 as well as activation and expression of MAPKs.

2. Materials and methods

2.1. Cell culture

Primary bronchial epithelial cells (PBECs) were obtained by protease digestion as described previously [18] from tracheobronchial tissue of 3 COPD patients with GOLD stage III and IV (undergoing lung transplantation), and 4 non-COPD lung donors of whom no further information was available. The study protocol was consistent with the Research Code of the University Medical Center Groningen (http://www.rug.nl/umcg/onderzoek/researchcode/index) and national ethical and professional guidelines ("Code of conduct; Dutch federation of biomedical scientific societies", http://www.federa.org).

Normal human bronchial epithelial cells from healthy donors (NHBE; Lonza group Ltd., Switzerland) and primary tracheobronchial epithelial cells (PBECs) from non-COPD lung donors and COPD patients were cultured in BEGM medium containing 1% penicillin and streptomycin, at 37 °C in a humidified 5% CO2 atmosphere. Cells were grown in 25 cm2 culture flasks coated with 0.1% fibronectin (Sigma-Aldrich, USA) and 3 mg/ml collagen (Inamed, USA) until they reached 90−95% confluency, and then 30000 cells per well were seeded on coated 24-well plates for experiments, except for MTS assay where 10000 cells were seeded on coated 96-well plates, grown to confluence and hormonally-deprived overnight before experimentation.

2.2. Treatments of cells

Cells were treated with low endotoxin recombinant human Hsp70 protein (rhHsp70) (Enzo Life Sciences, USA), in concentration range from 0.1 to 10 µg/ml, 2.5 and 15% of cigarette smoke extract (CSE), and combinations of CSE with rhHsp70. Endotoxin concentration in original solution of rhHsp70 was 2.7 EU/mg, determined by Limulus amebocyte lysate (LAL) test by manufacturer. Equivalent amount of LPS was used as negative control in all experiments.

2.3. Cigarette smoke extract (CSE)

We used Kentucky Research Cigarettes 3R4F (University of Kentucky, USA). Filters from 2 cigarettes were removed, and the smoke was bubbled through 25 ml of EMEM medium at constant rate (70 ml/min). This solution was considered 100% CSE and was freshly generated for each experiment. 100% CSE was diluted in BEBM medium containing 10 µg/ml transferrin, 5 µg/ml insulin and 1% penicillin, streptomycin and amphotericin B to final working concentrations (2.5% and 15%) and used within 1 h. CSE was standardized by measuring the absorbance at 320 nm [19].

2.4. Measurement of cytokine concentration

Cells were treated with rhHsp70, CSE or their combinations for 24 h. Cell-free supernatants were collected and stored at −80 °C until analysis.

The concentration of IL-6 was determined in supernatants by Pelikine Compact human IL-6 ELISA kit (IBL International, Germany), and IL-8 was determined by DuoSet ELISA kit (R&D Systems, USA), according to the manufacturer’s guidelines.

2.5. Expression of TLR2, TLR4 and Hsp70 mRNA

After the media for cytokine determination were removed, RNA was isolated from PBECs and NHBE cells by Trizol/chlorophorm method [20], using TRI reagent (Applied Biosystems, USA). cDNA was synthesized using RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, USA).

qPCR was performed using the 7300 Real Time PCR System, TaqMan Universal Master Mix and TaqMan Gene Expression Assays (Applied Biosystems, SAD): Hs02621280_s1 for tlr2; Hs00152939_m1 for tlr4; Hs00359163_s1 for Hsp70; and reference genes Hs99999907_m1 for β2-microglobulin (B2M) and Hs99999904_m1 for peptidil-prolil-izomerase A (PPIA).

2.6. Western blotting

NHBE cells were lysed with lysis buffer containing 50 mM Tris-HCl pH 8.0, 137 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM sodium-orthovanadate, and protease inhibitor (Complete Protease Inhibitor Cocktail Tablets, Roche) for 20 min on ice, and then centrifuged at 15000 g for 20 min at 4 °C. Protein concentration was determined using bicinchoninic acid (BCA) assay [21]. Samples were denatured by boiling for 5 min with 6x Laemmli sample buffer (0.375 M Tris−HCl pH 6.8, 12% w/v SDS, 3% v/v glycerol, 0.2% w/v bromophenol blue, 12% β-mercaptoethanol in distilled water) in 5:1 ratio.

30 µg of total protein was loaded onto a 10% polyacrylamide gel and run at 100 V. Transfer onto nitrocellulose membrane was
conducted at 250 mA for 90 min. Membranes were blocked for one hour with blocking buffer containing 5% skimmed milk in TBS + T (25 mM Tris pH 7.6, 150 mM NaCl, 0.05% Tween 20). Membranes were probed overnight at 4 °C with anti-phospho-ERK antibody (Phospho-p44/p42 MAPK (ERK1/2) (D13.14.4E) XP Rabbit mAb, #8454, Cell Signalling Technology, USA) diluted 1:2000 in blocking buffer; anti-ERK antibody (ERK 1 (C-16), #sc-93, Santa Cruz Biotechnology, USA) diluted 1:500 in 5% BSA in TBS; anti-phospho-JNK antibody (Phospho-SAPK/JNK (Thr183/Tyr185) Antibody, #9251, Cell Signalling Technology, USA) diluted 1:1000 in 5% BSA in TBS + T; anti-phospho-p38 antibody (Phospho-p38 MAPK (Thr180/Tyr182) (D3F9) XP® Rabbit mAb, #4511, Cell Signalling Technology, USA) diluted 1:1000 in 5% BSA in TBS + T; anti-p38 antibody (p38α (C-20); #sc-535; Santa Cruz Biotechnology, USA) in 5% BSA in TBS + T. A horseradish peroxidase-conjugated secondary antibody diluted 1:3000 (for anti-phospho antibodies) or 1:10000 (for MAPKs expression) in 5% skimmed milk in TBS + T was utilised to allow detection of the appropriate bands using chemiluminescence reagent containing 5 mg luminol (Sigma-Aldrich), 1 ml 1.5 M Tris-HCl pH 8.8, 14 ml ddH2O, 5 μl H2O2 and 150 μl enhancer (11 mg p-coumarin acid (Sigma-Aldrich) in 10 ml DMSO).

2.7. MTS assay

NHBE cells were treated with rhHsp70, CSE and their combinations for 24 h. 20 μl of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (Promega, USA) reagent was added into each well, and incubated for 3 h in a humidified 5% CO2 atmosphere. Absorbance was measured at 490 nm using a microplate reader (VICTOR3 1420 Multilabel counter, Perkin Elmer, USA).

2.8. Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM). Analysis of variance (ANOVA) followed by post hoc testing by Sidak method was used for all statistical analysis, except for calculations of interactions. When assessing combined effects of rhHsp70 with CSE, we compared our measured values with the calculated (expected) values [22]. The values were calculated as the mean value obtained after exposure to one substance alone (rhHsp70) plus the mean value obtained after exposure to another substance alone (CSE), as stated in the following equation:

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\text{SEM (expected for rhHsp70 + CSE) = (SEM for rhHsp70)}^2 + (\text{SEM for CSE})^2\]^{1/2}

Significant difference between expected (calculated) and measured values was determined using unpaired t-test. The results were as follows: a synergistic effect was implied if the measured values were significantly above the expected values; an antagonistic effect was implied if the measured values were significantly below the expected values.

The level of P < 0.05 was considered statistically significant for all analyses. Data were analyzed using GraphPad Prism 6.01 software (GraphPad Software Inc., USA).
Fig. 1. IL-6 and IL-8 secreted from NHBE cells (a, b; n = 5), PBEC non-COPD cells (c, d; n = 4), and PBEC COPD cells (e, f; n = 3) after 24 h treatment with rhHsp70. Data are presented as mean ± SEM. NT = non-treated cells. * statistically significant (P < 0.05) vs. NT.
combinations with rhHsp70. Again, the addition of rhHsp70 did not cause a further increase in IL-6 and IL-8 levels, and 1 μg/ml rhHsp70 even significantly reduced IL-8 secretion in the presence of 2.5% CSE (Fig. 3a and c). The calculated type of interaction for CSE and rhHsp70 was found to be antagonistic regarding IL-8 release (Fig. 3d).

Once again, significant and cell type-dependent differences in cytokines levels were observed after treatment with CSE alone or in combinations with rhHsp70, with non-COPD PBECs having the lowest concentrations in comparison with NHBE cells and PBECs from COPD patients.

Taking all together, whereas rhHsp70 alone did not significantly affect IL-6 and IL-8 secretion in NHBE cells, it further increased CSE-induced secretion of IL-6. In contrast, rhHsp70 alone significantly increased IL-6 and IL-8 release in PBECs, but did not further increase the CSE-induced release of IL-6 and IL-8. Moreover, antagonistic type of interactions for IL-8 secretion was found between CSE and rhHsp70 in PBECs from COPD patients.

3.3. Effects of eHsp70 and CSE on TLR2 and TLR4 mRNA expression

Since TLR2 and TLR4 are believed to be the main receptors for eHsp70, we treated NHBE cells, non-COPD PBECs and PBECs isolated from COPD patients with different concentrations of rhHsp70 (0.3 and 1 μg/ml), CSE (2.5 and 15%) and their combinations, and determined the expression of TLR2 and TLR4 mRNA (Fig. 4).

In NHBE cells and PBECs derived from lung donors there were no changes in mRNA expression for TLR2 or TLR4 after treatment with either CSE or rhHsp70 alone. In contrast, mRNA expression of TLR2 and TLR4 was significantly and strongly reduced in PBECs from COPD patients treated only with rhHsp70 or CSE, when compared to non-treated cells.

When combinations of rhHsp70 and CSE were applied on both NHBE cells and non-COPD PBECs, no differences in expression of TLR2 mRNA were found compared to non-treated cells. However, expression of TLR2 mRNA was significantly lower after treatment with combination of 2.5% CSE and 1 μg/ml rhHsp70 when

**Fig. 2.** Effect of CSE alone and in combination with rhHsp70 on IL-6 (a, c) and IL-8 (b, d) secretion from NHBE (a, b) and PBEC non-COPD cells (c, d) treated for 24 h. Data are presented as mean ± SEM. NT – non-treated cells. * statistically significant (P < 0.05) vs. NT. ** statistically significant (P < 0.05) vs. CSE alone.
compared to 2.5% CSE alone. On the other hand, the combination of 15% CSE and 0.3 mg/ml rhHsp70 caused significant increase in TLR4 mRNA expression compared to non-treated NHBE cells, but not to CSE alone. In addition, 2.5% CSE and 1 mg/ml rhHsp70 caused significantly lower expression of TLR4 mRNA compared to non-treated PBECs from lung donors, but also not to CSE alone.

In PBEC isolated from COPD patients combined application of CSE with 0.3 μg/ml rhHsp70 significantly raised TLR2 mRNA expression compared to CSE alone. In addition, TLR4 mRNA expression was significantly higher for the combination of 2.5% CSE and 0.3 μg/ml rhHsp70 than in non-treated cells and cells treated only with 2.5% CSE.

In summary, rhHsp70 and cigarette smoke alone down-regulated the expression of both TLR2 and TLR4 mRNA in PBECs from COPD subjects, but had no significant effect on those mRNAs in either NHBE cells or non-COPD PBECs. However, when applied together, combinations with lower rhHsp70 concentration significantly increased TLRs mRNA over the levels detected with CSE alone in PBEC COPD cells, while higher rhHsp70 concentration significantly decreased TLR2 mRNA expression in comparison with 2.5% CSE alone in both non-COPD PBECs and NHBE cells.

3.4. Effects of eHsp70 and CSE on the expression of Hsp70 mRNA

Intracellular Hsp70 has many cytoprotective functions, and might even counteract some pro-inflammatory effects. Therefore, we assessed the levels of its mRNA. In NHBE cells and PBECs from lung donors, there were no changes in mRNA expression of Hsp70 with individual CSE or rhHsp70 application. In contrast, mRNA expression of Hsp70 in PBEC cells from COPD patients treated with rhHsp70 or CSE alone was significantly lower compared to non-treated cells (Fig. 5).

In combined treatments, we found no differences in Hsp70 mRNA in NHBE cells and PBECs from COPD patients compared to non-treated cells or cells treated with CSE alone. However, in non-COPD PBECs, all combinations of rhHsp70 and CSE resulted in significantly lower expression of Hsp70 mRNA compared to non-treated cells and cells treated with CSE alone.

Altogether, we found no alterations in the expression of mRNA for Hsp70 in NHBE cells with applied treatments. In non-COPD
Fig. 4. Expression of mRNA for TLR2 (a, c, e) and TLR4 (b, d, f) in NHBE cells, PBEC non-COPD and PBEC COPD cells after 24 h treatment with rhHsp70, CSE or their combinations. Data are presented as mean ± SEM (n = 3). Dotted line represents expression of mRNA in non-treated cells set as 1. * statistically significant (P < 0.05) vs. NT. ** statistically significant (P < 0.05) vs. CSE alone.
PBECs, only combinations of CSE with rhHsp70 significantly modulated the level of Hsp70 mRNA, causing its down-regulation. In contrast, whereas CSE and rhHsp70 alone decreased the mRNA expression of Hsp70 in COPD-derived cells, this was no longer observed upon their combined treatments.

3.5. Activation of MAPKs with eHsp70 and CSE

Our data suggested that both eHsp70 and CSE influence pro-inflammatory cytokine production and alter TLR2/4 and intracellular Hsp70 expression, which might be related to MAPKs activation. Therefore, we detected MAPKs expression and activation by western blotting. Due to a restricted number of isolated PBECs, we used only NHBE cells for this set of experiments. As the number of NHBE cells was also limited to a certain extent, we only assessed treatments with rhHsp70 and CSE alone, not with their combinations, for different periods of time. We treated NHBE cells with rhHsp70 (0.3 or 1 μg/ml) or CSE (2.5% or 15%) for 30 min, 2 h and 8 h, based on the time-dependent increase in MAPKs phosphorylation by rhHsp70 and CSE in the NCI-H292 human bronchial epithelial cell line (data not shown).

We observed an increase in phosphorylation of p38 after treating NHBE cells with 15% CSE at 30 min, 2 h and 8 h (Fig. 6). However, rhHsp70 did not induce the activation of p38 MAPK. JNK1/2 (46 and 55 kDa, respectively) were significantly activated by 15% CSE after 30 min of treatment, while the 32–35 kDa c-Jun kinase band was detected at all times, and both rhHsp70 and CSE increased its activation after 30 min and 2 h compared to non-treated NHBE cells. Additionally, CSE caused increased activation of the 32–35 kDa c-Jun kinase after 8 h treatment.

Thus, higher CSE concentration activated JNK and p38 MAPKs, while eHsp70 and lower concentration of CSE increased activation of the 32–35 kDa c-Jun kinase only.

3.6. Cell viability

Inflammation and cell death are interconnected processes. We assessed cell viability with MTS test in NHBE cells only, for the above-mentioned reasons. We found that rhHsp70 and CSE alone or when used in combination did not significantly affect metabolic activity of NHBE cells (Fig. 7).

4. Discussion

Extracellular Hsp70 was found to be elevated in the blood of COPD patients [16,17], and smoking is one of the major risk factors for COPD [23]. In this study we hypothesized that inflammation-associated effects of eHsp70 increase the pro-inflammatory effects of cigarette smoke in airway epithelial cells, thus increasing its pathogenic effects. We compared the effects on primary airway...
epithelial cells from COPD patients and controls of different origin: commercially obtained NHBE cells and PBECs isolated from non-COPD lung donors. We observed that rhHsp70 has pro-inflammatory effects in PBECS from both COPD and non-COPD subjects. CSE increased pro-inflammatory effects in all cell types, and further increased it in combination with rhHsp70 in NHBE cells, an effect that may be related to the increased expression of TLR4. The same effect was not observed in PBECS. Moreover, we unexpectedly observed an antagonistic effect of rhHsp70 on CSE-induced IL-8 release specifically in PBECS derived from COPD patients, an effect that seems to be mediated independently from TLR4 expression and might be related to higher intracellular Hsp70 levels.

Several studies already confirmed pro-inflammatory effects of eHsp70 in different cells, including human bronchial epithelial cells [24–26]. Also, it is a well-known fact that cells exposed to cigarette smoke produce increased levels of pro-inflammatory cytokines, especially IL-8 [11,27–30]. Although some investigations have previously been conducted in relation to cigarette smoke effect on intracellular Hsp70, there is not much known about its interactions with eHsp70. Therefore, we treated cells with CSE together with rhHsp70. Best to our knowledge, our study is the first to show the effects of the simultaneous application of CSE and rhHsp70 on cells.

Our results show that rhHsp70 provoked an increase in IL-6 and IL-8 secretion in both non-COPD and COPD PBECS, with basal and rhHsp70-stimulated release of cytokines being stronger in PBECS from COPD patients. However, rhHsp70 did not further increase a release of cytokines after CSE treatment in PBECS, and even had an antagonistic effect on IL-8 release in COPD-derived PBECS. Contrary to this, rhHsp70 alone did not significantly affect IL-6 and IL-8 secretion in NHBE cells, but it further elevated CSE-induced secretion of IL-6 which could be related to the expression of TLR4. Similarly, it has previously been demonstrated that IL-8 secretion stimulated by CSE is reduced by antibodies to eHsp70 in human normal bronchial epithelial 16HBE cells and human lung fibroblasts, suggesting that eHsp70 promotes CSE-induced IL-8 secretion in those cells [11,12]. Previous studies demonstrated that the type of response to eHsp70 and CSE depends on origin of the cells and whether they are isolated from healthy individuals or COPD patients [31–36]. When we compared the cytokine levels among different primary lung epithelial cells, we noticed that non-stimulated NHBE cells secrete the highest basal concentrations of IL-6 (52.1 ± 9.6 pg/ml) compared to both non-COPD PBECS (7.0 ± 2.5 pg/ml) and PBECS from COPD patients (18.2 ± 6.3 pg/ml), while significantly more IL-8 is secreted from NHBE cells (1060.0 ± 133.8 pg/ml) compared to PBECS from lung donors (489.7 ± 148.3 pg/ml). In general, PBECS are more sensitive to rhHsp70 than NHBE cells, and the level of pro-inflammatory response was the strongest in COPD-derived PBECS. We also found that concentrations of IL-6 and IL-8 secreted from non-COPD PBECS after treatment with CSE are significantly lower compared to levels of IL-6 and IL-8 secreted from NHBE cells and PBECS isolated from COPD individuals. It is important to note that NHBE cells used in this study were isolated mostly from smokers (n = 4 smokers, n = 1 non-smoker). PBECS were from lung donors with normal lung function, where we do not have information about their smoking status, and PBECS from COPD patients originated from GOLD III and GOLD IV patients who were smokers or ex-smokers. Therefore, it is possible that the cause of different inflammatory response between those cells is, besides deregulation of inflammation in COPD, also their smoking status.

It is well-known that eHsp70 can activate immune system cells via TLR2 and TLR4 receptors, leading to the synthesis of pro-inflammatory cytokines [37–39]. Therefore, after observing rhHsp70-induced IL-6 and IL-8 secretion, next we assessed TLR2 and TLR4 mRNA expression. The expression of a DAMP gene set in epithelial cells from COPD patients was shown to be significantly stronger increased upon smoking compared to controls, with TLR2 and TLR4, through which eHsp70 signals, being among the core-enriched genes [40].

In our study, basal expression of mRNA for both TLR2 and TLR4 was significantly higher in PBECS from lung donors compared to NHBE cells (the lowest level of TLR2 mRNA) and COPD subjects-derived PBECS (the lowest level of TLR4 mRNA). Once again, current smoking (NHBE) and disease presence (PBECP COPD) might be of utmost influence on the cellular biological responses, including de novo synthesis of TLRs.

We showed that in the NHBE cells rhHsp70 alone did not affect pro-inflammatory responses which may be related to the relatively low expression of TLR2. The increase in IL-6 release upon the
combined stimulation with CSE and rhHsp70 was accompanied by increased TLR4 expression. In PBECs from non-COPD and COPD subjects, the addition of rhHsp70 did not increase CSE-induced cytokine secretion. In non-COPD PBECs, this was accompanied by a decrease in TLR2 and TLR4 expression, which may thus explain the lack of effect of the combination of stimuli on IL-6 secretion. In PBECs from COPD subjects, rhHsp70 and cigarette smoke alone down-regulated the expression of both TLR2 and TLR4 mRNA, but this was no longer observed upon the combination, leading to significantly increased TLRs mRNA over the levels detected with CSE alone, with especially strong increase in TLR4. This was not accompanied by an increase in IL-6 and even an antagonistic effect on IL-8 was observed, suggesting that additional mechanisms than TLR expression are involved in the regulation of these cytokines in COPD-derived cells upon the stimulation with rhHsp70 and CSE combination.

Several previous studies showed that eHsp70 can induce expression of TLR2 and TLR4 receptors [7,8], and that CSE influenced expression of TLR4 in human bronchial epithelial cells by increasing the synthesis of this receptor [41], directly activating TLR4, or indirectly causing cell damage and release of DAMPs molecules that bound to TLR4 [42]. On the other hand, CSE reduced mRNA and protein expression of TLR4 in human lung epithelial A549 cells, while TLR2 expression remained unchanged [43]. In addition, TLR receptors are significantly involved in exacerbations of COPD and are the primary mediators that activate the immune response to bacteria in the respiratory tract [3]. Decreased TLR2 expression was found in alveolar macrophages of COPD patients and healthy smokers [44], and CSE treatment of PBECs isolated from COPD patients resulted in reduced TLR4 receptor expression [45], which is in agreement with our results. TLR4 protective regulatory function was demonstrated against emphysematous changes provoked by smoking, and it was suggested that TLR4 receptor deregulation may contribute to the development of emphysema [46]. Therefore, deregulation of TLR2/4 receptors by either eHsp70 or CSE might have a significant impact on patients with COPD.

Increased cytokine release induced by eHsp70 or CSE could be related to the activation of MAPK signalling pathways through various receptors (not necessarily belonging to TLR family). In A549 cells, eHsp70 activated ERK, JNK, and p38 MAPK [47]. Conversely, Luo et al. showed that eHsp70 inhibits activation of MAPK signalling pathways in synovial fibroblasts [48].

We show that rhHsp70 did not elevate IL-6 and IL-8 release, and only activated 32–35 kDa c-Jun kinase in NHBE cells. It is possible that this kinase is involved in synthesis of other cytokines and/or in biological outcomes not related to cytokines when induced by eHsp70. Contrary to this, CSE provoked a significant increase in IL-6 and IL-8 secretion, and stimulated activation of c-Jun kinase, JNK1/2 and p38 MAPKs in NHBE cells, and these effects might be interconnected.

Previous study has found that ERK was activated in NHBE cells treated with CSE [49], which is not in agreement with our results. We detected ERK phosphorylation in CSE-treated NHBE cells, but its level of activation was similar to those in basal condition (non-treated cells). Also contrary to our results, another study on primary human airway epithelial cells showed that CSE did not activate MAPKs and did not affect the release of IL-8 [50].

We believe that a possible reason for discrepancies of those results is the variation in protocols for CSE preparation and/or interindividual variations of NHBE donors (their smoking history, age, sex, race, ethnicity etc.).

In general, it seems that intracellular Hsp70 has anti-inflammatory, while eHsp70 has pro-inflammatory effects. Whether the overall effects will be pro- or anti-inflammatory depends on the type of cell and the total inflammatory context as well as the concentration ratio of intracellular and extracellular Hsp70 [5]. Therefore, the expression of intracellular Hsp70 was also determined in this study. PBECs isolated from COPD patients had reduced Hsp70 mRNA expression after treatment with rhHsp70 or CSE alone, in contrast to NHBE cells and non-COPD PBECs. This effect was no longer observed upon their combined treatments, which was accompanied by an antagonistic effect on IL-8 secretion. This antagonistic effect in COPD could thus be related to the higher intracellular Hsp70 levels upon the combined treatment with rhHsp70 and CSE. It is also possible that this reduction of expression of Hsp70 in COPD-derived PBECs is due to exhaustion of mRNA synthesis for Hsp70 associated with increased cellular stress and damage in these cells, leading to impaired anti-inflammatory effects.

Some previous studies reported a decrease in Hsp70 mRNA and/or Hsp70 protein levels in the lymphocytes and total leukocytes of COPD patients [14,51]. The reduced binding of the heat shock factor 1 (HSF-1) to the heat shock element (HSE) might be the cause of the decrease in Hsp70 production. It was demonstrated that some MAPKs (ERK and JNK) could bind to and phosphorylate HSF-1, leading to suppression of its transcriptional activity [52,53]. This might be the scenario present in our COPD-derived PBECs. However, due to the restricted number of the isolated primary cells, MAPK signalling pathways were explored only in commercially obtained NHBE cells and not in PBECs in this study, but this would be interesting to investigate in the future.

In conclusion, both eHsp70 and CSE can stimulate pro-inflammatory responses in primary airway epithelial cells, and their interactions could provoke a modulation of inflammation in those cells. Antagonistic effects of eHsp70 and CSE in COPD-derived cells might be a consequence of their desensitization to stimuli in order to dampen the ongoing inflammation in COPD. In turn, this might lead to reduced capacity to properly respond to the noxious stimuli, such as bacteria or viruses, thus leading to adverse consequences for patients with COPD.

Conflicts of interest

The authors declare that they have no conflict of interest.

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