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ORIGINAL PAPER

Effect of cigarette smoke and dexamethasone on Hsp72 system of alveolar epithelial cells

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Abstract Smoking is the leading risk factor of chronic obstructive pulmonary disease (COPD) and lung cancer. Corticosteroids are abundantly used in these patients; however, the interaction of smoking and steroid treatment is not fully understood. Heat shock proteins (Hsps) play a central role in the maintenance of cell integrity, apoptosis and cellular steroid action. To better understand cigarette smoke-steroid interaction, we examined the effect of cigarette smoke extract (CSE) and/or dexamethasone (DEX) on changes of intracellular heat shock protein-72 (Hsp72) in lung cells. Alveolar epithelial cells (A549) were exposed to increasing doses (0; 0.1; 1; and 10 μM/μl) of DEX in the medium in the absence(C) and presence of CSE. Apoptosis, necrosis, Hsp72 messenger-ribonucleic acid (mRNA) and protein expression of cells were measured, and the role of Hsp72 on steroid effect examined. CSE reduced the number of viable cells by significantly increasing the number of apoptotic and necrotic cells. DEX dose-dependently decreased the ratio of apoptosis when CSE was administered, without change in necrosis. CSE−DEX co-treatment dose-dependently increased Hsp72 mRNA and protein expression, with the highest level measured in CSE+DEX (10) cells, while significantly lower levels were noted in all respective C groups. Pretreatment with Hsp72 silencing RNA confirmed that increased survival observed following DEX administration in CSE-treated cells was mainly mediated via the Hsp72 system. CSE significantly decreases cell survival by inducing apoptosis and necrosis. DEX significantly increases Hsp72 mRNA and protein expression only in the presence of CSE resulting in increased cellular protection and survival. DEX exerts its cell protective effects by decreasing apoptotic cell death via the Hsp72 system in CSE-treated alveolar epithelial cells.

Keywords Hsp72 · Cigarette smoke · COPD · Emphysema · Lung cancer · Dexamethasone

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Introduction

Smoking is the largest preventable health problem worldwide, causing five million deaths yearly (WHO 2006). In respect of the lungs, the most important smoking-related diseases are chronic obstructive pulmonary disease (COPD) and lung cancer, both representing high morbidity and mortality illnesses. Increasing body of evidence indicates association between COPD, especially emphysema, and lung cancer mainly through the common risk factor cigarette smoke (Punturieri et al. 2009 and Ueda et al. 2006).

Cigarette smoke contains more than 4,000 chemicals, which could have toxic, mutagenic, and carcinogenic effects. It

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contains 10^{14}–10^{16} free radicals/puff, disturbing the oxidant–antioxidant balance leading to cellular damage in the lungs. It has been shown that in response to cigarette smoke, apoptosis and necrosis of the alveolar wall cells occurs (Liu et al. 2009). This destruction results in progressive cell loss and airway enlargement: the prominent features of emphysema (Demedts et al. 2006). Cigarette smoke consists of more than 4,000 compounds, so it is difficult to determine which components are the main contributors to cellular damage. Studies assessing the deleterious effects of cigarette smoke are mostly using different forms of cigarette smoke extracts. Cigarette smoke mediated oxidative stress and inflammatory events in the airway and alveolar epithelium are important processes in the pathogenesis of smoking-related pulmonary diseases (Faux et al. 2009 and Kode et al. 2006). Tobacco smoke initiates apoptosis in airway epithelial cells as a result of mitochondrial damage. This effect appears to result mainly from free radical activity in tobacco smoke and not from nicotine (Ramage et al. 2006).

Heat shock proteins (Hsps) are highly conserved proteins throughout evolution. The heat shock protein-70 (Hsp70) family proteins are molecular chaperones with an essential role in the assembly of polypeptides. They play a central role in refolding disrupted proteins, thereby limiting cellular injury and restoring cellular function. The cellular expression of the inducible form of the Hsp70 family (Hsp72) is increased following various forms of stress like heat, ischemia, oxidative stress, heavy metal irons, radiation as well as exposure to various cytokines (Hartl 1996).

The linkage between Hsps and apoptosis is widely studied. It is known, that the Hsps exert anti-apoptotic effects by inhibiting the release of the cytocrome *c* from the mitochondria. Hsp72 inhibits caspase-9 and other caspases, as well as the extrinsic pathway of apoptosis (Xanthoudakis and Nicholson 2000; Powers et al. 2009).

Steroids are commonly used drugs for many acute and chronic pulmonary inflammatory diseases, including asthma, COPD and lung cancer. The therapeutic effects of these agents have been mainly attributed to their anti-inflammatory and immunosuppressive effect. Corticosteroids elicit apoptosis in inflammatory cells (Melis et al. 2002). In contrast, they protect mammary gland and intestinal epithelial cells against apoptotic cell death (Feng et al. 1995). However, it is not clear yet, how steroids affect lung parenchyma or airway epithelium.

Steroids are stress hormones and during cellular stress increase in Hsp72 might be necessary to elicit proper glucocorticoid action. It is well known, that a heat shock protein 90(Hsp90)/Hsp70-based multiprotein chaperone machinery is necessary for the prompt function of the glucocorticoid receptor (GR). It plays an important role in the opening of the ligand-binding cleft of the GR, in the translocation to the nucleus, both in GR movement to transcription regulatory sites and in the disassembly of regulatory complexes as the hormone level declines (Pratt and Toft 2003). It also plays a critical role in stabilization of the GR to ubiquitylation and proteasomal degradation. There are recent data that the initial GR interaction with Hsp70 appears to be critical for the triage between Hsp90 heterocomplex assembly and preservation of receptor function. It is possible that all physiologically significant actions of Hsp90 require the Hsp70-dependent assembly of client protein-Hsp90 heterocomplexes (Pratt et al. 2006).

Taking into account that cigarette smoke has an effect on alveolar epithelial cells, we examined the effect of cigarette smoke extract (CSE) on alveolar epithelial cell stress and cell death in an in vitro setting. As Hsp72 plays a key role in apoptosis and in the protection against cellular injury, its function in the process was examined. As steroids are widely used in clinical practice (including smokers), the interaction of CSE and dexamethasone (DEX) on apoptosis and cellular Hsp72 function was also assessed.

Methods

Culture of A549 human alveolar epithelial cells

The A549 human type II alveolar epithelial cell line (ECACC No: 86012804) was obtained from the European Collection of Cell Cultures (Sigma-Aldrich Co., Budapest, Hungary). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 mg/ml glucose and supplemented with 10% fetal bovine serum (FBS; Biochrome AG., Berlin, Germany), 1% antibiotic–antimycotic solution (AB; Sigma-Aldrich Co., Budapest, Hungary) and 2 mmol/L L-glutamine (Biochrome AG, Berlin, Germany) in a humidified incubator with 5% CO_2 at 37°C. After confluency, cells were trypsinized and used for experiments. Cell number for cell plating was counted by trypan blue exclusion assay.

Preparation of CSE

Cigarette smoke extract was freshly prepared for each experiment and supplemented with 10% FBS immediately before use. CSE was prepared by a modification of the method of (Bernhard et al. 2004). In brief, CSE was prepared by bubbling the smoke from two commercially available filter cigarettes (Marlboro; Philip Morris Products, Hungary, EU; nicotine 0.8 mg, tar 10 mg) through 16 ml of pre-warmed (37°C) serum-free cell culture medium. The cigarettes were machine smoked at a rate of 35 ml over a time period of 2 s followed by a pause of 28 s before repeating, matching the smoking habits of an average smoker. The resulting CSE was applied to epithelial cell cultures within 30 min of preparation. Using this method, preliminary experiments confirmed, that 24-h incubation

with the undiluted CSE extract resulted in significant decrease in survival of A549 cells. Medium for control cells was treated similarly, without the use of cigarettes.

Stability of CSE solutions was checked using high-performance liquid chromatography-mass spectrometry (HPLC-MS/MS) methodology. Solutions were deproteinized using acetonitrile precipitation. Precipitate was sedimented by centrifugation, and 1 ml of supernatant was pipetted into an Eppendorf tube and evaporated to dryness. Sample was reconstituted in acetonitrile/water 5:95 containing 1% aceticacid. Five-microliter sample was injected onto HPLC column (Purospher STAR RP-18, 55 mm× 2.1 mm×3 μm) and was eluted using gradient elution starting formacetonitrile/water 5:95, ending at 95:5. Positive ion electrospray mass spectrometry was used as detection method. Molecular ion of nicotine at *m*/*z* 163 and cotinine at m/z 177 were monitored. Nicotine and cotinine concentrations were found to be within 10% of median concentrations for all samples.

Treatment groups

Twenty-four hours before the treatment A549 cells were plated on tissue culture-treated 6-well plates (Costar, Corning Incorporated; Corning, NY, USA) in 2-ml DMEM with 10% FBS and 1% AB at concentrations of 6.5×10^5/2 ml to reach 80–90% confluence at the beginning of the experiment. CSE with 10% FBS and 1% AB and dexamethasone (dexaratiopharm 4 mg, Ratiopharm, Hungary) at three different concentrations: 0.1, 1, and 10 μM/μl was given to medium. DEX free (0 μM) groups served as steroid naïve controls (C). The cells were treated with CSE and/or DEX for 24 h at 37°C in a humidified atmosphere containing 5% CO2.

Preliminary results confirmed that following different incubation times (24 and 48 h) and using undiluted and 2× and 10× diluted samples, 24-h incubation and non-diluted CSE resulted in significant decrease in cell numbers under control (no dexamethasone treatment) conditions.

Treatment groups are listed in Table 1.

Cell count and viability

Cell number in vials following 24 h treatment was measured using Cell-Dyn 3200 (Abbott Laboratories Ltd, Budapest, Hungary). Apoptosis was assessed by annexin V/propidium iodide staining using flow cytometric (FACS) analysis as described previously (Chen et al. 2008a).

FACS analysis of Hsp72-positive cells

Hsp72 expression was analyzed using the following protocol: cell pellets were washed with 0.5 ml PBS, incubated with 0.5 ml FACS ™ Permeabilizing Solution2(10×) (Perm2; BD Bioscience Co., Soft Flow Hungary Kutato Fejleszto Kft., Pecs, Hungary) for 10 min in the dark, room temperature (RT). After permeabilisation cells were centrifuged (800×*g*/RT/7 min) and incubated with 2-μl anti-Hsp72 antibody (rabbit anti-human Hsp72 IgG obtained from Dr. L. László, Eötvös University, Budapest, Hungary) for 30 min, RT, in the dark. Cells were washed with 0.5 ml Perm2 and centrifuged (800×*g*/RT/5 min) and incubated with 1-μl Cy5-conjugated secondary antibody (Cy5-conjugated AffiniPure $F(ab')_2$ Fragment Goat anti-rabbit IgG (H+L) antibody, Jackson ImmunoResearch Laboratories Inc., Izinta Ltd., Budapest, Hungary) for 30 min RT, in the dark. Negative controls were incubated with the Cy5-conjugated secondary antibody only. Cells were washed with 0.5 ml Perm2, centrifuged (800×*g*/RT/5 min) and resuspended in 500-μl PBS. The cytometric analysis was carried out using a FACSAria cytometer (Becton Dickinson, San Jose, CA, USA). Thirtythousand cells were collected and results were analyzed using the BD FACSDiva Software (Becton Dickinson, San Jose, CA, USA).

RNA isolation and real-time reverse transcription polymerase chain reaction

Reverse transcription polymerase chain reaction (RT-PCR) was performed as previously described (Rusai et al. 2009). Briefly, total ribonucleic acid (RNA) was isolated from the cells by RNeasy RNA isolations kit (Qiagen GmbH, Hilden Germany) according to the instructions of the manufacturer. The quality and quantity of the RNA were spectrophotometrically confirmed. One-microgram RNA was reverse transcripted using SuperScript II RNase H^- (Invitrogen Corp. Csertex Kft., Budapest, Hungary) to generate first-strand complementary dezoxy-ribonucleic acid (cDNA). Hsp72 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression were determined by real-time RT-PCR quantification using Syber Green on a Light Cycler system (Roche Diagnostics, Mannheim, Germany). The PCRs were performed in 196-well plates in a final volume of 20 μl. We used 10 μl of Brilliant II Fast syBR QPCR Master Mix (Stratagene, Biomedica Hungary), 7 μl of destilled water, 1–1 μl of the primers and 1 μl of cDNA sample. The PCRs were carried out using the following conditions: one cycle at 95°C for 2 min., followed by cycles at 95°C for 20 s and 60°C for 40 s, and one cycle at 95°C for 30 s and 45°C for 30 s. The sequences of the specific primer pairs for Hsp72 and GAPDH are presented in Table 2. After each Light Cycler run, PCR products were separated by electrophoresis on 2.5% agarose gels and visualized by staining with ethidium bromide. In order to control the length of the generated PCR products, a 100-bp DNA ladder (Invitrogen Corp. Carlsbad, CA, USA) was used. Quantification was performed with second derivative method by monitoring the cycle

Table 1 Treatment groups

C	DMEM+10% FBS+1% AB
C+DEX (0.1)	DMEM+10% FBS+1% AB+0.1 μM/μl dexamethasone
C+DEX (1)	DMEM+10% FBS+1% AB+1 μM/μl dexamethasone
C+DEX (10)	DMEM+10% FBS+1% AB+10 μM/μl dexamethasone
CSE	Cigarette smoke extract+10% FBS+1%AB
CSE+DEX (0.1)	Cigarette smoke extract+10% FBS+1% AB+0.1 μM/μl dexamethasone
CSE+DEX (1)	Cigarette smoke extract+10%FBS+1% AB+1 μM/μl dexamethasone
CSE+DEX (10)	Cigarette smoke extract+10%FBS+1% AB+10 μM/μl dexamethasone
scr-RNA	DMEM+10% FBS+1% AB+transfected with scrambled silencing RNA (as negative transfection control)
siRNA	DMEM+10% FBS+1% AB+transfected with Hsp72 silencing RNA

number at which the fluorescent sign could be distinguished from the background (crossing point). Serially diluted cDNA samples were used as external standards. Results were analyzed by using Light Cycler software version 3.5.3 (Roche Diagnostics, Mannheim, Germany).

Hsp72 silencing—SiRNA transfection

For transfection siPORT™ *Neo FX*™ Transfection Agent (Ambion, AM4511; Applied Biosystems, Hungary) was used according to the instructions of the manufacturer. All transfections were carried out in triplicate. To transfect, 2.5 μl of 10 nmol silencing RNA (siRNA) was diluted in 25-μl DMEM into each well, 1.25-μl NeoFx (Ambion, Inc.) in 25-μl DMEM was added to each sample, incubated for 10 min, at RT. Fifty microliters of diluted transfection mixture was given to wells that already contained the inhibitors and incubated for another 10 min at RT, 450 μl of diluted cell suspension mixture containing 2.2×10^5 cells was added on top of the complex. After 24 h the medium was changed with DMEM containing 10% FBS and 1% AB. Scrambled (Scr)-RNA was used similarly as negative control for the transfection. After the next 24 h cells were treated with CSE, DEX, or both. The samples were assayed by flow cytometry (BD FACSaria, San Jose, CA, USA) 24 h thereafter measuring apoptosis, necrosis and Hsp72 protein expression as described previously.

Statistical analysis

Data were analysed using Statistica version 7 software (StatSoft, Inc., Tulsa, OK, USA). After testing the normality with Shapiro–Wilk's test, the non-parametric Mann–Whitney *U* test was used to determine the levels of difference among all treatment groups. *p* Values less than 0.05 were considered statistically significant. All data are expressed as mean±standard deviation.

Results

Cell number after the treatments

Under control conditions cells proliferated, reaching about $8.55\pm1.1\times10^5$ final cell number/vial. Following DEX treatment no significant change in cell number was noted. In contrast, CSE treatment significantly reduced the cell number 24 h after incubation as compared to controls (Fig. 1.). CSE+DEX co-treatment dose-dependently and significantly increased the total cell count as compared to CSE treatment alone, reaching similar number in both DEX (10) groups (C+DEX (10): $10.22\pm0.77\times10^5$ cell/vial; CSE+DEX (10): $8.86\pm0.49\times10^5$ cell/vial).

Apoptosis

DEX slightly decreased the number of the apoptotic cells in controls, reaching statistical significance only in the C+DEX (10) group. In steroid-naïve CSE-treated cells, apoptosis tripled as compared with the steroid naïve controls. DEX treatment significantly reduced apoptosis in all CSE-treated groups, abolishing the difference between CSE-treated and respective control groups (Fig. 2.).

Table 2 Nucleotide sequence of specific primer pairs applied for the real-time detection of heat shock protein-72 (Hsp72) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

Gene	Primer pairs and probes	Product length	Annealing
Hsp72	Forward: 5′-TGC GAG AGG GCC AAG AGG AC-3′ Reverse: 5′-GTC GCG CCC GTT GAA GAA GT-3′	276 bp	60°C
GAPDH	Forward: 5′-GTC AGT GCC GGC CTC GTC TCA TAG-3′ Reverse: 5′-TCG CGC TCC TGG AAG ATG GTG AT-3′	265 bp	60°C

bp base pairs

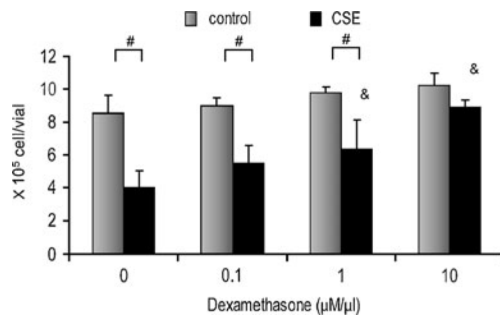

Fig. 1 Cell number after the treatments. A549 alveolar epithelial cells were treated with increasing doses of dexamethasone (DEX) in the medium: 0 (steroid naïve), 0.1, 1, and 10 μM/μl in the absence (*controls*) and presence of cigarette smoke extract (*CSE*). Cell number/vial after 24 h of incubation was measured. CSE decreased the number of the cells following 24-h incubation. DEX treatment dose-dependently increased survival of CSE-treated cells, reaching similar numbers in both DEX (10)-treated groups. (& $p<0.05$ vs. 0 μM/μl DEX; # $p<0.05$ vs. control)

Necrosis

The ratio of necrotic cells did not differ in controls, whether DEX treatment was used or not (C, 3.2±0.63%; C+DEX (0.1), 2.95±0.36%; C+DEX (1), 3.2±1.39%; C+DEX (10), 3.08±1.32%). In contrast, CSE significantly increased the number of necrotic cells. Similarly to controls, DEX had no additional effect on necrosis in CSE-treated cells resulting in significantly higher necrotic cell rate in all CSE groups (CSE, 6.24±1.02%; CSE+DEX (0.1), 6.12±2.11%; CSE+DEX (1), 6.68±1.1%; CSE+DEX (10), 4.7±0.75%; $p<0.05$ vs. respective control group).

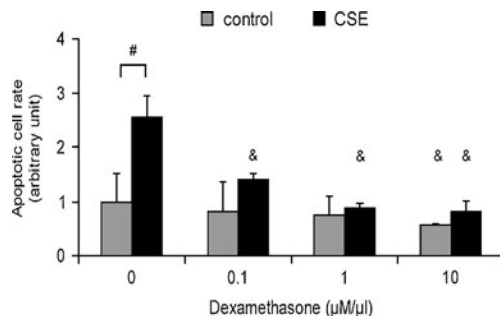

Fig. 2 Apoptosis. A549 alveolar epithelial cells were treated with increasing doses of dexamethasone (DEX) in the medium: 0 (steroid naïve), 0.1 μM/μl, 1, and 10 μM/μl in the absence (*controls*) and presence of cigarette smoke extract (*CSE*). Apoptosis following 24 h of incubation was measured by annexin V/propidium iodide staining using flow cytometric analysis. Highest dose of DEX decreased the number of apoptotic cells under control conditions. CSE significantly increased apoptosis, whereas CSE-DEX co-treatment decreased the number of apoptotic cells in a dose-dependent manner. Apoptosis in the steroid naïve control group is expressed as 1, relative changes are presented for all other groups (arbitrary unit). (& $p<0.01$ vs 0 μM/μl DEX; # $p<0.05$ vs control)

Hsp72 mRNA expression

Hsp72 mRNA expression did not change following DEX treatment in controls (Fig. 3a). Administration of CSE alone was associated with low level of Hsp72 mRNA, similar to the level observed in steroid naïve controls. In contrast, DEX treatment resulted in significant increase in Hsp72 mRNA, already using 0.1 μM/μl concentration in the presence of CSE, and further significant increase was observed using 1 μM/μl DEX. However, no further increase was detected in the CSE+DEX (10) cells (Fig. 3b).

Hsp72 protein expression

In steroid naïve controls Hsp72 protein expression of individual epithelial cells was low, similarly to the ratio of Hsp72 expressing cells (Fig. 4a and b).

DEX treatment decreased both the ratio of Hsp72 expressing cells and the cellular Hsp72 content of the cells significantly in controls.

In all CSE-treated groups, the ratio of Hsp72-positive cells was significantly higher as compared to respective controls ($p<0.05$). DEX treatment significantly and dose-dependently increased the number of Hsp72 expressing cell, with the highest ratio measured in the CSE+DEX (10) group, where nearly 80% of cells were expressing the protein. CSE treatment significantly increased the cellular Hsp72 protein content in all (steroid naïve and DEX treated) groups as compared to respective controls. The mean intensity of Hsp72 in Hsp72-positive cells increased with increasing doses of DEX following CSE treatment, with significantly increased values in DEX (1) and DEX (10) groups compared to DEX (0) group.

Hsp72 siRNA

Transfection with siRNA was successful in steroid naïve controls and CSE-treated cells. As previous experiments confirmed the highest Hsp72 expression in CSE+DEX (10) cell, this group was chosen as the third group, to assess the effect of CSE and steroid co-treatment on cellular Hsp72 expression in this experimental setting.

While scr-RNA did not change cellular Hsp72 protein expression, siRNA treatment resulted in significant decrease of cellular Hsp72 protein under control and CSE-treated conditions (Fig. 5a, b). Similarly, Hsp72 protein expressing cell number significantly decreased in all groups following siRNA treatment. In C and CSE groups, siRNA reduced the number of Hsp72-positive cells by 80% and 60%, respectively, while it reached 75% in the CSE+DEX (10) group. The most marked decrease in Hsp72 was registered in the CSE+DEX (10) group following siRNA treatment, where significant reduction in the ratio of Hsp72-

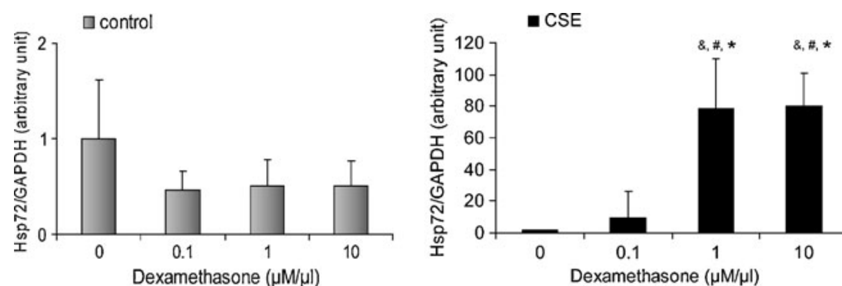

Fig. 3 Hsp72 mRNA expression. A549 alveolar epithelial cells were treated with increasing doses of dexamethasone (DEX) in the medium: 0 (steroid naïve), 0.1, 1, and 10 μM/μl in the absence (*controls*) and presence of cigarette smoke extract (*CSE*). Hsp72mRNA expression was determined by real-time PCR. Optical density of Hsp72 was corrected for that of GAPDH. DEX treatment alone did not change Hsp72 mRNA expression, while in the presence of CSE DEX administration was associated with significant increase in Hsp72 mRNA expression. Expression of Hsp72 mRNA in the steroid naïve control group is expressed as 1; relative changes are presented for all other groups (arbitrary unit). (&$p<0.01$ vs 0 μM/μl DEX; #$p<0.05$ vs control; *$p<0.01$ vs 0.1 μM/μl DEX)

positive cells, and the reduction of the content of the expressing cells was observed abreast.

Parallel to the decreased expression of Hsp72 protein in siRNA treated cells, apoptosis significantly increased in all groups, indicating a direct link between cellular Hsp72 and apoptotic cell death (Fig. 6.).

Discussion

Cigarette smoke significantly reduces survival and proliferation of A549 alveolar epithelial cells under in vitro conditions. CSE-induced severe injury resulted in increased apoptosis and necrosis of cells. Previous data by Kaushik et al. also showed that CSE at high concentration kills lung cells by increasing reactive oxygen species production and apoptosis. In contrast, at low concentration CSE-induced cell proliferation and DNA synthesis via activation of p38 mitogen-activated protein kinase in A549 lung alveolar cells (Kaushik et al. 2008). Besides toxicity induced cell destruction, CSE might also inhibit cell proliferation (Jiao et al. 2006). Liu et al. demonstrated that cigarette smoke decreased the proliferation of alveolar cells, partly by induction of pro-inflammatory cytokines resulting in inflammation that contributed to alveolar cell damage (Liu et al. 2009).

In our experiments, we used immortalized alveolar epithelial cells, the main cell type present in alveoli. The human cell line A549 is used to study the physiology of the alveolar type (AT) II cells (Forbes and Ehrhardt 2005). This cell type constitutes about 60% of alveolar epithelial cells and about 15% of all lung parenchymal cells, while they cover less than 5% of the alveolar air spaces of adult human lungs. They play multi-functional role in alveolar homeostasis by forming the alveolar barrier, producing surfactants and proliferating and differentiating into AT I cells as a repair/replacement mechanism. The destruction of these cells by CSE is important in the development of emphysema (Demedts et al. 2006). On the other hand A549 cells are immortalized, carrying characteristics of cancer cells. There are several studies that demonstrate the linkage between COPD and the risk of lung cancer (Punturieri et al.

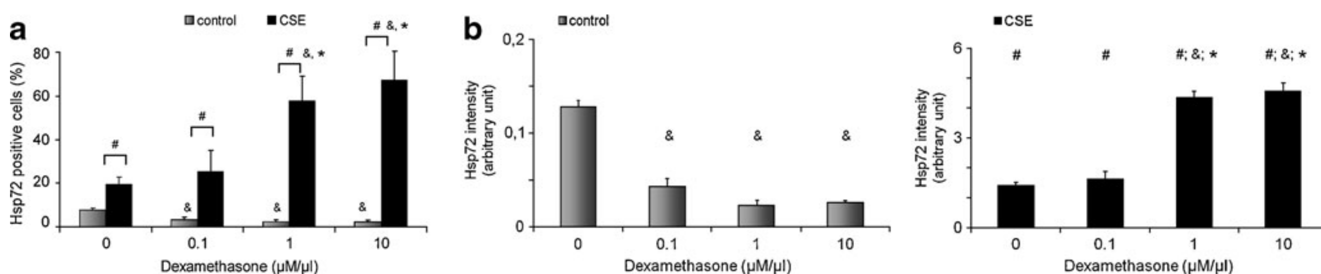

Fig. 4 Hsp72 protein expression: percentage of Hsp72-positive cell (**a**) and intracellular amount/intensity of Hsp72 (**b**). A549 alveolar epithelial cells were treated with increasing doses of dexamethasone (DEX) in the medium: 0 (steroid naïve), 0.1, 1, and 10 μM/μl in the absence (*controls*) and presence of cigarette smoke extract (*CSE*). Hsp72 protein expression was determined by flow cytometric analysis using rabbit anti-human Hsp72 IgG primary antibody and rabbit Cy5-conjugated secondary antibody. The ratio of Hsp72 expressing cells and the intracellular Hsp72 intensity of cells were measured. DEX treatment decreased Hsp72 protein expression in controls, whereas co-treatment with CSE and DEX increased the Hsp72 protein expression dose dependently. In all CSE-treated groups, Hsp72 protein expression was significantly higher as in respective control groups, with the highest difference measured in DEX (10) cells. Data for protein levels of Hsp72 were obtained by computerized analysis of the FACS. (&$p<0.01$ vs 0 μM/μl DEX; #$p<0.01$ vs control; *$p<0.01$ vs 0.1 μM/μl DEX)

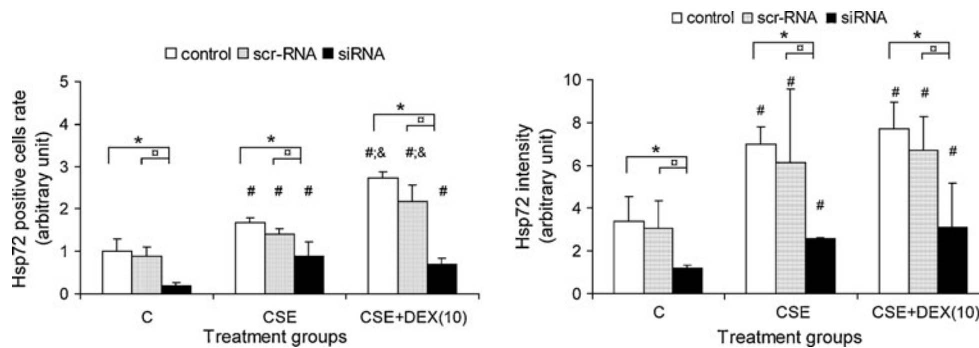

Fig. 5 Hsp72 protein expression after siRNA treatment: percentage of Hsp72-positive cells (**a**) and intracellular amount/intensity of Hsp72 (**b**). Hsp72 silencing RNA (siRNA) was used in control (**c**), cigarette smoke extract (*CSE*)-treated and CSE plus dexamethasone 10 μM/μl (DEX (10) co-treated A549 alveolar epithelial cells. Scrambled (Scr)-RNA was used similarly as negative control for the transfection. After silencing, Hsp72 protein expression was measured by flow cytometry. In all groups, siRNA effectively suppressed Hsp72 expression. In **a** steroid-naïve control, values (*C*) are expressed as 1; relative changes are presented for all other groups (arbitratry unit). (&$p<0.01$ vs CSE-treated groups; #$p<0.05$ vs steroid-naïve control groups; *$p<0.01$ vs. control and ¤$p<0.01$ vs. scr groups)

2009; Ueda et al. 2006). COPD is characterized by inflammation and alveolar wall destruction, while lung cancer is caused by uncontrolled proliferation of cells. Favoring the importance of lung parenchyma destruction, Houghton et al. demonstrated that even mild emphysema confers a substantial risk of developing lung cancer (Houghton et al. 2008).

Under control conditions steroid treatment did not affect apoptosis, necrosis or cellular Hsp72 mRNA or protein expression of alveolar epithelial cells. In contrast, steroid treatment significantly increased cell survival and proliferation in the presence of CSE by significantly and dose-dependently decreasing apoptosis, without influencing necrosis.

Programmed cell death mechanism may contribute to lung cell loss and tissue injury following exposure to cigarette smoke; however, their role in the pathogenesis of COPD remains unclear (Henson et al. 2006). Examination of lung tissues from COPD patients revealed increased number of apoptotic cells than observed in normal or in smokers without COPD lungs (Yokohori et al. 2004). Autophagy represents a cellular adaptive mechanism that promotes survival under various stress condition including nutrient deprivation, cytokines, and oxidative stress (Levine and Yuan 2005). Autophagy was demonstrated to be increased in lung tissue of COPD patients, especially in the early phase of the disease (Ryter and Choi 2010). Similarly, CSE-treated lung cells also demonstrated increase in autophagy (Chen et al. 2008b). Autophagy can influence several forms of cell death, including caspase-independent cell death and apoptosis (Boya et al. 2005). CSE might also have induced autophagy in our setting; however, the accurate relationship between autophagy and programmed cell death remains unclear.

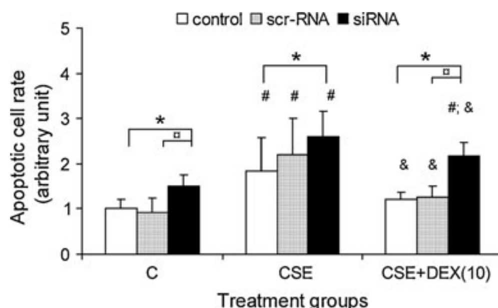

Fig. 6 Apoptosis following Hsp72 siRNA treatment. Hsp72 silencing RNA (siRNA) was used in control (*C*), cigarette smoke extract (*CSE*) treated and CSE plus dexamethasone 10 μM/μl (DEX (10) co-treated A549 alveolar epithelial cells. Scrambled (Scr)-RNA was used similarly as negative control for the transfection. After silencing Hsp72 apoptosis was measured by annexin V/propidium iodide staining using flow cytometric analysis. Decrease in Hsp72 protein after siRNA treatment was associated with significant increase of apoptotis following CSE treatment. Apoptosis in the steroid naïve control group (*C*) is expressed as 1; relative changes are presented for all other groups (arbitrary unit). (&$p<0.01$ vs CSE-treated groups; #$p<0.05$ vs steroid naïve control groups; *$p<0.01$ vs. control and ¤$p<0.01$ vs. scr groups)

Most COPD patients are current or ex-smokers. Extensive evidence suggests that susceptibility/resistance to smoking-induced lung disorders is genetically determined (Ammous et al. 2008). Clinically, unlike asthma, COPD is considered as a mainly steroid resistant inflammatory disorder, however in severe cases steroids are suggested for the treatment of these patients (GOLD 2010). The interaction between cigarette smoke and glucocorticoids is not well understood. Failure to respond may result from reduced binding to the intracellular glucocorticoid receptor, decreased receptor expression, enhanced activation of inflammatory pathways, or lack of co-repressor activity. These events can be modulated by oxidative stress, T helper type 2 cytokines, or high levels of inflammatory mediators, all of which may lead to a worsened clinical outcome (Adcock and Barnes 2008; Barnes 2010)

There are controversial data about the effect of steroids on human cells. Glucocorticoids act as pro-apoptotic drugs for

inflammatory cells, but in epithelial cells it seems to have anti-apoptotic effect (Melis et al. 2002; Feng et al. 1995). Dorscheid et al. demonstrated that in cultured airway epithelium corticosteroids induce apoptosis (Dorscheid et al. 2006), on the other hand there are data showing that steroids may inhibit apoptosis induced by Fas ligation in alveolar epithelial cells (Wen et al. 1997).

Our data provide first evidence for upregulation of Hsp72 in alveolar epithelial cells exposed to CSE. While not affecting cellular Hsp72 mRNA level, Hsp72 protein content of the individual cells, and the ratio of Hsp72 staining cells increased significantly after CSE treatment. One possible mechanism could include the activation of cell preservation mechanism, including decreased degradation of Hsp72 in cells exposed to severely damaging substances. Heat shock protein induction acting in a cytoprotective manner by preventing the onset of apoptosis is extensively studied. Hsp72 has been shown to protect cells both from apoptosis and necrosis (Fekete et al. 2006; McConkey 1998). In the literature, there is sparse evidence confirming other noxa—for example dimethylarsinic acid exposure—elevating intracellular Hsp72 levels, changing the localization of the molecule and suppressing apoptosis of human alveolar cells (Kato et al. 2000). Previous data in myocytes showed that DEX treatment dose-dependently increased the expression of Hsp72 via activation of heat shock factor (Sun et al. 2000).

DEX treatment increased alveolar epithelial cell survival in the presence of CSE. Hsp72 siRNA abolished the mRNA and protein increase in CSE-DEX cells, in parallel apoptosis increased, less cells survived. These results confirm upregulation of Hsp72 in the presence of CSE in order to insure cell survival, and indicate key protecting role for Hsp72 under this cellular stress condition. Elevated intracellular HSP levels are known as the stress response. Following various forms of stress, trimerization and nuclear translocation of cytoplasmatic heat shock factor-1 occurs, binds with the heat shock element and consequently transcription of HSP genes follows. In parallel, intracellular HSP is known to be cytoprotective and induces the cell's anti-apoptotic mechanisms. It represses gene expression, modulates cell-cycle progression and has anti-inflammatory effects (Li and Srivastava 2004; Hartl 1996; Xanthoudakis and Nicholson 2000; Powers et al. 2009). On the other hand, HSPs can be secreted to the extracellular milieu playing important role as a danger signal for the immune system under stress. Extracellular HSPs stimulate pro-inflammatory cytokine synthesis and enhance anti-tumor surveillance (Malusecka et al. 2008; Michils et al. 2001).

Surface expression of Hsp72 has been previously reported mainly on human tumor cell lines. Cells of the innate immune system: macrophages, dendritic cells and NK cells, are involved in the surveillance of functionally aberrant cells through the recognition of a specific C-terminal structure of Hsp72 a danger signal in living cells (Tani et al. 2009). The expression of Hsp72 on the cell surface and release into the circulation might increase immune activity and subsequent cell damage in lung tissues following cigarette smoke exposure. Further experiments in animals or human lung tissues are needed to address this question.

According our results, steroid treatment increases survival and proliferation only in CSE-treated alveolar cells. There are only sparse clinical data assessing the effects of long-term corticosteroid treatment in COPD or lung cancer smoker patients. Shaker et al. demonstrated that long-term inhaled corticosteroid (budesonide) showed a trend towards reducing the progression of emphysema in current smokers suffering from COPD (Shaker et al. 2009). Also the fact that patients with COPD who are treated with inhaled corticosteroids and quit smoking have reduced incidence of lung cancer and death suggests that inhibiting alveolar cell apoptosis and subsequent inflammation might retard tumor development (Kiri et al. 2009).

Glucocorticoid receptor (GR) function is dependent on the Hsp90/Hsp70 chaperone machinery. Initial GR interaction with Hsp70 appears to be critical for the triage between Hsp90 heterocomplex assembly and preservation of receptor function. Hsp70 is required for the assembly of protein-Hsp90 heterocomplexes, and the two chaperones interact directly with each other while opening the steroid binding cleft in the GR (Pratt and Toft 2003). According to our experiments, increase in the inducible form of Hsp70 (Hsp72) following CSE administration might enable proper action of administered DEX, by increasing the assembly of GR and opening the steroid binding cleft. However, further studies are needed to assess CSE-induced changes at the GR level.

In conclusion, our data confirmed that CSE induces apoptosis and necrosis in alveolar epithelial cells. DEX reduces CSE-induced cellular damage, by decreasing apoptosis. This is the first evidence of DEX-CSE interaction showing a key role of Hsp72 in alveolar epithelial cell survival. Our siRNA experiments confirmed that elevated Hsp72 is essential in the observed anti-apoptotic and protective effects of DEX following CSE exposure. Hsp72 might represent a new key molecule and a potential therapeutic target in smoke exposed lung cells. As millions of smokers are treated with glucocorticoids new data on cigarette smoke and glucocorticoid interaction are needed. Future experiments are necessary to evaluate the role of Hsp72 in smoker and non-smoker COPD patients, especially assessing the effects on alveolar destruction.

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References

Adcock IM, Barnes PJ (2008) Molecular mechanisms of corticosteroid resistance. Chest 134(2):394–401, Review

Ammous Z, Hackett NR, Butler MW, Raman T, Dolgalev I, O'Connor TP, Harvey BG, Crystal RG (2008) Variability in small airway epithelial gene expression among normal smokers. Chest 133 (6):1344–1353

Barnes PJ (2010) Mechanisms and resistance in glucocorticoid control of inflammation. J Steroid Biochem Mol Biol 120(2–3):76–85

Bernhard D, Huck CW, Jakschitz T, Pfister G, Henderson B, Bonn GK, Wick G (2004) Development and evaluation of an in vitro model for the analysis of cigarette smoke effects on cultured cells and tissues. J Pharmacol Toxicol Methods 50(1):45–51

Boya P, González-Polo RA, Casares N, Perfettini JL, Dessen P, Larochette N, Métivier D, Meley D, Souquere S, Yoshimori T, Pierron G, Codogno P, Kroemer G (2005) Inhibition of macro-autophagy triggers apoptosis. Mol Cell Biol 25(3):1025–1040

Chen NY, Lai HH, Hsu TH, Lin FY, Chen JZ, Lo HC (2008a) Induction of apoptosis in human lung carcinoma A549 epithelial cells with an ethanol extract of Tremella mesenterica. Biosci Biotechnol Biochem 72(5):1283–1289

Chen ZH, Kim HP, Sciurba FC, Lee SJ, Feghali-Bostwick C, Stolz DB, Dhir R, Landreneau RJ, Schuchert MJ, Yousem SA, Nakahira K, Pilewski JM, Lee JS, Zhang Y, Ryter SW, Choi AM (2008b) Egr-1 regulates autophagy in cigarette smoke-induced chronic obstructive pulmonary disease. PLoS ONE 3 (10):e3316

Demedts IK, Demoor T, Bracke KR, Joos GF, Brusselle GG (2006) Role of apoptosis in the pathogenesis of COPD and pulmonary emphysema. Respir Res 7:53

Dorscheid DR, Patchell BJ, Estrada O, Marroquin B, Tse R, White SR (2006) Effects of corticosteroid-induced apoptosis on airway epithelial wound closure in vitro. Am J Physiol Lung Cell Mol Physiol 291(4):L794–L801

Faux SP, Tai T, Thorne D, Xu Y, Breheny D, Gaca M (2009) The role of oxidative stress in the biological responses of lung epithelial cells to cigarette smoke. Biomarkers 14(Suppl 1):90–96

Fekete A, Vannay A, Vér A, Rusai K, Müller V, Reusz G, Tulassay T, Szabó AJ (2006) Sex differences in heat shock protein 72 expression and localization in rats following renal ischemia-reperfusion injury. Am J Physiol Renal Physiol 291(4):F806–F811

Feng Z, Marti A, Jehn B, Altermatt HJ, Chicaiza G, Jaggi R (1995) Glucocorticoid and progesterone inhibit involution and programmed cell death in the mouse mammary gland. J Cell Biol 131(4):1095–1103

Forbes B, Ehrhardt C (2005) Human respiratory epithelial cell culture for drug delivery applications. Eur J Pharm Biopharm 60(2):193–205

GOLD. Available at: http://www.goldcopd.com Accessed 20 June 2010

Hartl FU (1996) Molecular chaperones in cellular protein folding. Nature 381(6583):571–579, Review

Henson PM, Vandivier RW, Douglas IS (2006) Cell death, remodeling, and repair in chronic obstructive pulmonary disease? Proc Am Thorac Soc 3(8):713–717, Review

Houghton AM, Mouded M, Shapiro SD (2008) Common origins of lung cancer and COPD. Nat Med 14(10):1023–1024

Jiao ZX, Ao QL, Xiong M (2006) Cigarette smoke extract inhibits the proliferation of alveolar epithelial cells and induces apoptosis. Sheng Li Xue Bao 58(3):244–254, PubMed PMID: 16786109

Kato K, Yamanaka K, Nakano M, Hasegawa A, Okada S (2000) 72-kDa stress protein (hsp72) induced by administration of dimethylarsinic acid to mice accumulates in alveolar flat cells of lung, a target organ for arsenic carcinogenesis. Biol Pharm Bull 23(10):1212–1215

Kaushik G, Kaushik T, Khanduja S, Pathak CM, Khanduja KL (2008) Cigarette smoke condensate promotes cell proliferation through disturbance in cellular redox homeostasis of transformed lung epithelial type-II cells. Cancer Lett 270(1):120–131

Kiri VA, Fabbri LM, Davis KJ, Soriano JB (2009) Inhaled corticosteroids and risk of lung cancer among COPD patients who quit smoking. Respir Med 103(1):85–90

Kode A, Yang SR, Rahman I (2006) Differential effects of cigarette smoke on oxidative stress and proinflammatory cytokine release in primary human airway epithelial cells and in a variety of transformed alveolar epithelial cells. Respir Res 7:132

Levine B, Yuan J (2005) Autophagy in cell death: an innocent convict? J Clin Invest 115(10):2679–2688, Review. Erratum in: J Clin Invest. 2006 Dec;116(12):3293

Li Z, Srivastava P. Heat-shock proteins. Curr Protoc Immunol. 2004 Feb;Appendix 1:Appendix 1T. Review.

Liu Y, Gao W, Zhang D (2009) Effects of cigarette smoke extract on A549 cells and human lung fibroblasts treated with transforming growth factor-beta1 in a coculture system. Clin Exp Med 10 (3):159–167

Malusecka E, Krzyzowska-Gruca S, Gawrychowski J, Fiszer-Kierzkowska A, Kolosza Z, Krawczyk Z (2008) Stress proteins HSP27 and HSP70i predict survival in non-small cell lung carcinoma. Anticancer Res 28(1B):501–506

McConkey DJ (1998) Biochemical determinants of apoptosis and necrosis. Toxicol Lett 99(3):157–168, Review

Melis M, Siena L, Pace E, Gjomarkaj M, Profita M, Pirazzoli A, Todaro M, Stassi G, Bonsignore G, Vignola AM (2002) Fluticasone induces apoptosis in peripheral T-lymphocytes: a comparison between asthmatic and normal subjects. Eur Respir J 19(2):257–266

Michils A, Redivo M, Zegers de Beyl V, de Maertelaer V, Jacobovitz D, Rocmans P, Duchateau J (2001) Increased expression of high but not low molecular weight heat shock proteins in resectable lung carcinoma. Lung Cancer 33(1):59–67

Powers MV, Clarke PA, Workman P (2009) Death by chaperone: HSP90, HSP70 or both? Cell Cycle 8(4):518–526

Pratt WB, Toft DO (2003) Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. Exp Biol Med (Maywood) 228(2):111–133

Pratt WB, Morishima Y, Murphy M, Harrell M (2006) Chaperoning of glucocorticoid receptors. Handb Exp Pharmacol 172:111–138, Review. PubMed PMID: 16610357

Punturieri A, Szabo E, Croxton TL, Shapiro SD, Dubinett SM (2009) Lung cancer and chronic obstructive pulmonary disease:needs and opportunities for integrated research. J Natl Cancer Inst 101 (8):554–559

Ramage L, Jones AC, Whelan CJ (2006) Induction of apoptosis with tobacco smoke and related products in A549 lung epithelial cells in vitro. J Inflamm (Lond) 3:3

Rusai K, Prókai A, Szebeni B, Fekete A, Treszl A, Vannay A, Müller V, Reusz G, Heemann U, Lutz J, Tulassay T, Szabó AJ (2009) Role of serum and glucocorticoid-regulated kinase-1 in the protective effects of erythropoietin during renal ischemia/reperfusion injury. Biochem Pharmacol 79(8):1173–1181

Ryter SW, Choi AM (2010) Autophagy in the lung. Proc Am Thorac Soc 7(1):13–21, Review

Shaker SB, Dirksen A, Ulrik CS, Hestad M, Stavngaard T, Laursen LC, Maltbaek N, Clementsen P, Skjaerbaek N, Nielsen L, Stoel B, Skovgaard LT, Tonnesen P (2009) The effect of inhaled corticosteroids on the development of emphysema in smokers assessed by annual computed tomography. COPD 6(2):104–111

Sun L, Chang J, Kirchhoff SR, Knowlton AA (2000) Activation of HSF and selective increase in heat-shock proteins by acute

dexamethasone treatment. Am J Physiol Heart Circ Physiol 278 (4):H1091–H1097

Tani F, Ohno M, Furukawa Y, Sakamoto M, Masuda S, Kitabatake N (2009) Surface expression of a C-terminal alpha-helix region in heat shock protein 72 on murine LL/2 lung carcinoma can be recognized by innate immune sentinels. Mol Immunol 46 (7):1326–1339

Ueda K, Jinbo M, Li TS, Yagi T, Suga K, Hamano K (2006) Computed tomography-diagnosed emphysema, not airway obstruction, is associated with the prognostic outcome of early-stage lung cancer. Clin Cancer Res 12(22):6730–6736

Wen LP, Madani K, Fahrni JA, Duncan SR, Rosen GD (1997) Dexamethasone inhibits lung epithelial cell apoptosis induced by IFN-gamma and Fas. Am J Physiol 273(5 Pt 1):L921–L929

WHO. Available at: (www.wpro.who.int/media_centre/fact_sheets/fs_20060530.htm). Accessed 20 June 2010

Xanthoudakis S, Nicholson DW (2000) Heat-shock proteins as death determinants. Nat Cell Biol 2(9):E163–E165, Review

Yokohori N, Aoshiba K, Nagai A, Respiratory Failure Research Group in Japan (2004) Increased levels of cell death and proliferation in alveolar wall cells in patients with pulmonary emphysema. Chest 125(2):626–632