

## Research Paper

# Cigarette smoke induces endoplasmic reticulum stress response and proteasomal dysfunction in human alveolar epithelial cells

Anita Somborac-Baćura<sup>1,2</sup>, Marco van der Toorn<sup>1</sup>, Lorenza Franciosi<sup>3</sup>, Dirk-Jan Slebos<sup>4</sup>, Tihana Žanić-Grubišić<sup>2</sup>, Rainer Bischoff<sup>3</sup> and Antoon J. M. van Oosterhout<sup>1</sup>

<sup>1</sup>Laboratory of Allergology and Pulmonary Diseases, University Medical Center Groningen, GRIAC Research Institute, University of Groningen, Groningen, The Netherlands

<sup>2</sup>Department of Medical Biochemistry and Hematology, Faculty of Pharmacy and Biochemistry, University of Zagreb, Zagreb, Croatia

<sup>3</sup>Department of Analytical Biochemistry, Center for Pharmacy, University of Groningen, Groningen, The Netherlands

<sup>4</sup>Department of Pulmonary Diseases, University Medical Center Groningen, GRIAC Research Institute, University of Groningen, Groningen, The Netherlands

## New findings

- What is the central question of this study?

The endoplasmic reticulum stress response caused by cigarette smoke may lead to excessive apoptosis with disruption of the epithelial barrier, thus contributing to chronic obstructive pulmonary disease. One way of promoting cell survival is to facilitate degradation of cigarette smoke-induced protein damage through the ubiquitin–proteasome pathway. Direct effects of gas-phase cigarette smoke on proteasomal activities have not been demonstrated previously.

- What is the main finding and what is its importance?

We show that cigarette smoke induces protein damage and triggers the endoplasmic reticulum stress response in human alveolar epithelial cells. A significant reduction of all three proteasomal activities was found. Ineffective degradation of damaged proteins could lead to a sustained epithelial stress response and development of chronic obstructive pulmonary disease.

Cigarette smoking is the major risk factor for chronic obstructive pulmonary disease. Cigarette smoke (CS) causes oxidative stress and severe damage to proteins in the lungs. One of the main systems to protect cells from the accumulation of damaged proteins is the ubiquitin–proteasome pathway. In the present study, we aimed to find out whether exposure of alveolar epithelial cells to CS induces an endoplasmic reticulum (ER) stress response by accumulation of damaged proteins that are inefficiently degraded by the proteasomes. The hypothesis was tested in a human alveolar epithelial cell line (A549) exposed to gas-phase CS. Exposure to gas-phase CS for 5 min caused an increase in the amount of ubiquitin–protein conjugates within 4 h. Cigarette smoke exposure also induced the ER stress response marker eIF2 $\alpha$ , followed by a significant reduction of nascent protein synthesis and increase in the level of free intracellular amino acids. Moreover, CS exposure significantly reduced all three proteasomal activities (caspase-, trypsin- and chymotrypsin-like activity) within 4 h, which was still present after 24 h. It can be concluded that gas-phase CS induces ER stress in A549 alveolar epithelial cells, leading to inadequate protein turnover caused by an accumulation of damaged proteins, reduction in nascent protein synthesis and inhibition of the proteasome. We suggest that prolonged ER stress may lead to excessive

A. Somborac-Baćura and M. van der Toorn contributed equally to this work.

**cell death with disruption of the epithelial barrier, contributing to development of chronic obstructive pulmonary disease.**

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**Corresponding author** A. J. M. van Oosterhout: Laboratory of Allergology and Pulmonary Diseases, University Medical Center Groningen, Hanzplein 1, 9713 GZ Groningen, The Netherlands. Email: a.j.m.van.oosterhout@path.umcg.nl

The adverse effects of cigarette smoke (CS) on human health are well established; however, there is still incomplete understanding of the mechanisms by which CS leads to the development of smoking-related diseases, such as chronic obstructive pulmonary disease (COPD). Cigarette smoke contains numerous harmful compounds, including free radicals, oxidants and reactive oxygen and nitrogen species (Church & Pryor, 1985; Pryor & Stone, 1993). It is well established that these species contribute to oxidative damage of proteins inside the lung (Wright *et al.* 1994). Oxidatively modified proteins tend to aggregate and accumulate in the cell, potentially disturbing normal cellular functions and initiating cell death (Sherman & Goldberg, 2001; Poppek & Grune, 2006; Min *et al.* 2011).

In eukaryotic cells, the endoplasmic reticulum (ER) is an organelle responsible for protein synthesis, proper folding, post-translational modifications and quality control. However, different stressful conditions, including oxidative stress, can result in the accumulation of misfolded or unfolded proteins inside the ER, a condition known as ER stress (Kelsen *et al.* 2008; Todd *et al.* 2008). The ER attempts to restore protein homeostasis by activating a group of signal transduction pathways collectively termed the unfolded protein response (UPR). The UPR promotes cell survival by reducing accumulation of aberrantly folded proteins in the ER through a decrease in protein translation, an increase in production of chaperone proteins and enhancement of protein degradation by the ubiquitin–proteasome pathway, in a process referred to as ER-associated degradation (Hoseki *et al.* 2010). If the UPR fails to resolve ER stress, the cell becomes functionally compromised, and the UPR may initiate programmed cell death (apoptosis; Shore *et al.* 2011; Walter & Ron, 2011).

The ubiquitin–proteasome pathway is one of the main systems to protect cells from misfolded and damaged proteins (Sherman & Goldberg, 2001). The majority of proteins destined for proteasomal degradation are first covalently attached to a polymeric chain of ubiquitin molecules (Shang & Taylor, 2011). Subsequently, ubiquitinated proteins are unfolded by the 26S proteasome and hydrolysed into small peptides with the release of reusable ubiquitin (Sherman & Goldberg, 2001; Glickman & Ciechanover, 2002; Poppek & Grune, 2006; Shang & Taylor, 2011). The 26S proteasome is a large, multicatalytic protease composed of a 20S core particle that carries the

catalytic activity and two regulatory 19S particles. The 20S core particle is a barrel-shaped structure composed of two identical outer  $\alpha$ -rings and two identical inner  $\beta$ -rings. The catalytic sites are localized at the  $\beta 1$  (caspase-like activity),  $\beta 2$  (trypsin-like activity) and  $\beta 5$  (chymotrypsin-like activity) subunits (Orlowski & Wilk, 2000; Glickman & Ciechanover, 2002; Friguet, 2006). Most of the peptide products generated by the proteasome are rapidly hydrolysed into amino acids by downstream proteases and aminopeptidases. Free amino acids cannot be stored. These amino acids are reused for protein synthesis or for the increased metabolic demands of the oxidatively stressed cells (Grune *et al.* 1997).

Recent studies show a possible role of proteostasis imbalance and CS-induced ER stress response in the pathophysiology of COPD (Jorgensen *et al.* 2008; Kelsen *et al.* 2008; Malhotra *et al.* 2009; Geraghty *et al.* 2011; Min *et al.* 2011). Our previous study showed that gas-phase CS causes an increase in the intracellular level of most free amino acids inside the A549 alveolar epithelial cells (Abello *et al.* 2008). A possible explanation for this phenomenon was that CS caused intensive ubiquitination of damaged proteins, leading to proteasomal degradation and generation of free amino acids. Based on these data, in this work we examined the direct effect of gas-phase CS on the accumulation of ubiquitinated proteins and proteasomal activities. Furthermore, we hypothesized that inappropriate function of this process might lead to the activation of ER stress response. Our data show an overall disturbance of intracellular protein homeostasis due to the CS-induced ER stress response caused by accumulation of damaged proteins that are inefficiently degraded by the proteasomes.

## Methods

### Cell culture

The alveolar type II epithelial adherent cell line (A549) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The A549 cell line was established from human alveolar cell carcinoma, but has characteristic features of the normal type II alveolar epithelial cells (Lieber *et al.* 1976) and is widely used as a model for studying the effects of cigarette smoke on the lungs. Cells were grown in RPMI 1640 media (BioWhittaker, Verviers, Belgium) supplemented with

10% heat-inactivated fetal calf serum (BioWhittaker) and 100 U ml<sup>-1</sup> penicillin and streptomycin (BioWhittaker). Cells were grown in 25 cm<sup>2</sup> plastic culture flasks (Costar, Cambridge, MA, USA) at 37°C in a humidified incubator maintained at an atmosphere of 95% air and 5% CO<sub>2</sub> until 80–90% confluence was reached. Before the experiments, cells were incubated for 16 h in serum-free media.

### Exposure of alveolar epithelial cells to CS

Alveolar epithelial cells were exposed to air (negative control) or gas-phase CS as described previously (van der Toorn *et al.* 2007b). Briefly, before the exposure, cell culture medium was removed and the culture flask positioned upside down, allowing direct contact of CS with epithelial cells. Kentucky 3R4F research-reference cigarettes were smoked using a peristaltic pump. Immediately before the experiments, filters were cut off and cigarettes were smoked for exactly 1 or 5 min at a rate of 8 l h<sup>-1</sup>. Cigarette smoke was directly distributed inside the culture flasks. After the exposure of cells to gas-phase CS or air, cell culture medium was placed back on the cells and cell cultures were returned to the incubator for a specific period until they were harvested for analyses. The advantage of using the gas-phase CS, rather than the CS extract that is more commonly used for *in vitro* studies, is that direct distribution of CS over the cell culture more closely resembles the process of inhaling gaseous CS into human lungs and its distribution over the respiratory tract surface during cigarette smoking.

### Immunodetection by Western blotting

Ubiquitination of damaged proteins and phosphorylation of the  $\alpha$ -subunit of the eukaryotic initiation factor-2 (eIF2 $\alpha$ ) were analysed by Western blotting. Cells were treated with CS for 1 or 5 min and then returned to the incubator and cultured for the specified period. As positive controls, cells were exposed to 1  $\mu$ M epoxomicin (Sigma-Aldrich, Zwijndrecht, The Netherlands), an inhibitor of proteasomal activity, or 2 mM DTT (Sigma-Aldrich), an ER stress-inducing agent (Groll & Huber, 2004; Wang *et al.* 2011). After the incubation, medium was removed and total cell lysates were prepared. Proteins were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. The immunodetection of ubiquitinated proteins (P4D1; sc-8017),  $\beta$ -actin (C4; sc-47778; Santa Cruz Biotechnology, Santa Cruz, CA, USA), phosphorylated (9721) and total eIF2 $\alpha$  (9722; Cell Signaling Technology, Danvers, MA, USA) was performed according to the manufacturer's guidelines by chemoluminescence detection (ECL, Amersham, Little Chalfont, UK) using an FX imager (Bio-Rad Laboratories, Veenendaal, The Netherlands).

### Measurement of protein synthesis

Cells were treated with CS for 1 or 5 min and then returned to the incubator and cultured for 4 h. Cycloheximide, an inhibitor of protein synthesis, served as a positive control (final concentration 10  $\mu$ M). Nascent protein synthesis in cultured A549 cells was assayed using the Click-iT reaction cocktail (Invitrogen, Breda, The Netherlands). Briefly, after incubation the cells were washed with prewarmed L-methionine-free RPMI medium (Invitrogen). Washed cells were resuspended in prewarmed L-methionine-free RPMI medium containing 25  $\mu$ M L-azidohomoalanine, the amino acid analogue of methionine (Invitrogen). Cells were returned to the incubator and cultured for another 4 h. After the incubation, cells were washed with PBS and detached with trypsin–EDTA (Invitrogen). The cell suspension was fixed with 4% paraformaldehyde (Sigma-Aldrich) for 15 min followed by permeabilization with 0.25% Triton X-100 (Sigma-Aldrich) in PBS for 20 min. After permeabilization, cells were washed with 3% bovine serum albumin (Sigma-Aldrich) in PBS and resuspended in Click-iT reaction cocktail for 30 min at room temperature, protected from light. After the incubation the cells were washed with 3% bovine serum albumin in PBS and analysed using a FACSCalibur flow cytometer (Becton Dickinson Medical Systems, Heidelberg, Germany).

### Quantification of free amino acids by liquid chromatography–mass spectrometry using stable isotope labelling

Amino acids were labelled with pentafluorophenyl-activated esters of poly(ethylene glycol) (PEG-OPFP) derivatives and analysed by liquid chromatography–mass spectrometry (LC-MS) according to the method of Abello *et al.* (2008). Briefly, CS-treated cells were detached with cell dissociation buffer (Invitrogen), followed by three wash cycles, resuspension in 1 ml of 100 mM sodium phosphate (pH 8), 1 mM EDTA and finally lysed by sonication. Insoluble material was removed by centrifugation at 20,000g for 45 min at 4°C. Each sample was labelled with 10  $\mu$ l of 5 mM PEG-OPFP in DMSO solution. The PEG-OPFP labels contained zero, two, six or eight <sup>13</sup>C atoms for the samples exposed to air, epoxomicin, CS or epoxomicin plus CS, respectively. Samples were divided into four groups according to the type of exposure, and 20  $\mu$ l of a randomly selected sample from each of the four groups was mixed and then analysed by nano-LC-MS (Agilent Technologies Netherlands B.V., Amstelveen, The Netherlands), using a chip–cube interface (Agilent Technologies, catalogue no. G4240-62002). Integration of all peaks was automated using QuantAnalysis 1.8/Bruker Daltonics DataAnalysis 3.4 (Bruker Daltonik GmbH, Bremen, Germany). Peaks were defined by their *m/z*

values  $\pm 0.5$  Da based on singly charged states and their retention times  $\pm 0.5$  min by comparison to a standard solution of derivatized amino acids. Peak integration was checked manually in selected cases. The values of the peak areas for each amino acid (MS response) were normalized with respect to the protein concentration.

### Measurement of proteasomal activities using the fluorogenic substrates

Cells were treated with CS for 1 or 5 min and then returned to the incubator and cultured for another 4 or 24 h. As a positive control, cell lysates were exposed to  $1 \mu\text{M}$  epoxomicin, an inhibitor of proteasomal activity. Proteasomal activities were measured as described previously (Verdoes *et al.* 2006). Briefly,  $25 \mu\text{g}$  total protein lysate was added to  $100 \mu\text{l}$  substrate buffer, containing 20 mM Hepes (pH 8.2), 0.5 mM EDTA, 1% DMSO, 1 mM ATP (Sigma-Aldrich) and  $50 \mu\text{M}$  Z-Leu-Leu-Glu-AMC (caspase-like activity),  $50 \mu\text{M}$  Ac-Arg-Leu-Arg-AMC (trypsin-like activity) or  $50 \mu\text{M}$  Suc-Leu-Leu-Val-Tyr-AMC (chymotrypsin-like activity). The fluorogenic substrates Z-Leu-Leu-Glu-AMC (caspase-like activity) and Suc-Leu-Leu-Val-Tyr-AMC (chymotrypsin-like activity) were purchased from Calbiochem (Merck Biosciences, Darmstadt, Germany). The fluorogenic substrate Ac-Arg-Leu-Arg-AMC (trypsin-like activity) was purchased from Boston Biochem Inc. (Cambridge, MA, USA). The released fluorescent group was measured every 30 s for 15 min at  $37^\circ\text{C}$  using a FL600 fluorescent plate reader (Bio-Tek Instruments, Abcoude, The Netherlands; with an excitation wavelength of 360 nm and emission wavelength of 460 nm). The average change of fluorescence over 15 min was used to calculate specific proteasomal activities of each sample.

### Measurement of lactate dehydrogenase

Lactate dehydrogenase activity can be used as an indicator of cell membrane integrity and serves to assess cytotoxicity resulting from cigarette smoke. Cells were treated with CS for 1 or 5 min and then returned to the incubator and cultured for another 24 h. After the incubation period, cell culture medium was collected and centrifuged at  $1000g$  for 5 min at  $4^\circ\text{C}$ . Lactate dehydrogenase activity was determined using the CytoTox-ONE assay kit (Promega, Leiden, The Netherlands) and a FL600 fluorescent plate reader (Bio-Tek Instruments, Abcoude, The Netherlands). Lactate dehydrogenase was determined according to the manufacturer's instructions.

### Data analysis

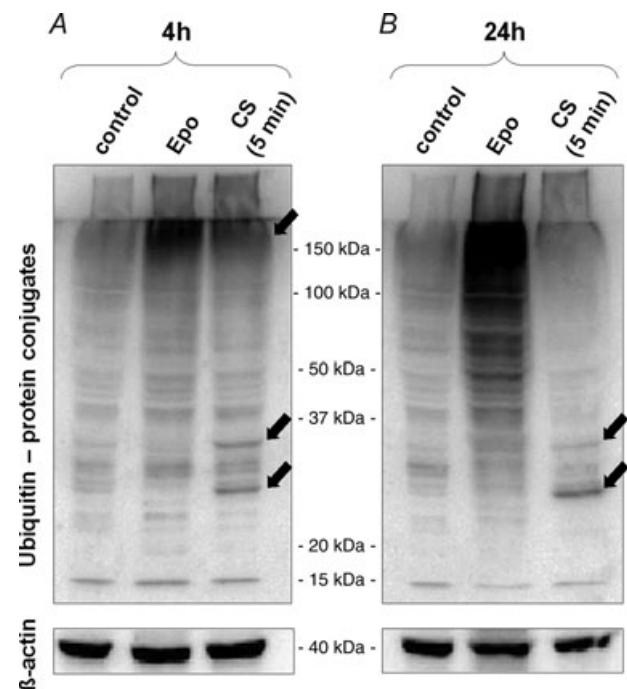
Western blot analyses were performed in three independent experiments. All other experiments

(presented with numerical results in graphs) were carried out with either three or four samples for each set of conditions ( $n = 3$  or  $n = 4$ ) as stated next to each graph. Calculations were performed using the Prism 4 for Windows (GraphPad Software Inc., San Diego, CA, USA). Comparisons between different experimental groups were performed with Dunnett's multiple comparison tests. A value of  $P < 0.05$  was considered significant. Results are presented as the means  $\pm$  SEM.

## Results

### Cigarette smoke increases ubiquitin–protein conjugates in alveolar epithelial cells

We measured the level of ubiquitin–protein conjugates by gel electrophoresis and immunoblotting with anti-ubiquitin antibodies. Figure 1 shows an increase in the amount of ubiquitin–protein conjugates in the lysates of epithelial cells exposed to CS for 5 min and subsequently cultured for the next 4 or 24 h. After 4 h (Fig. 1A) we observed more intensive bands of ubiquitin–protein

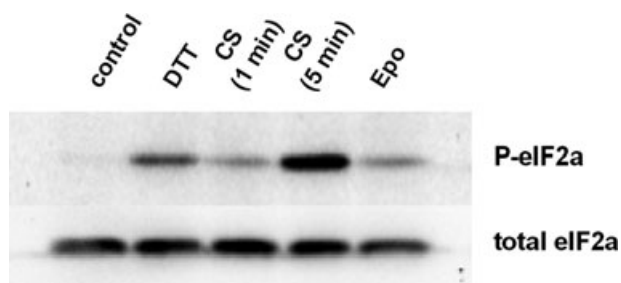


**Figure 1. Cigarette smoke (CS) increases the amount of ubiquitin–protein conjugates in alveolar epithelial cells** Western blot analysis of ubiquitin–protein conjugates in A549 cells exposed to CS for 5 min followed by culture for 4 (A) or 24 h (B). Control cells were exposed to air (instead of CS) for 5 min (negative control). Epoxomicin (Epo), an inhibitor of proteasomal activity, was used as a positive control. Arrows indicate bands with an increase in intensity. The experiment was carried out for three independent replicates; a representative one is shown.

conjugates with molecular masses around 30 and 35 kDa as well as those above 150 kDa, compared with the air control. After 24 h (Fig. 1B) the ubiquitin–protein conjugates above 150 kDa were less intense, but the ubiquitin–protein conjugates with molecular masses around 30 and 35 kDa remained clearly visible. Treatment with 1  $\mu\text{M}$  epoxomicin (an inhibitor of proteasomal activity) resulted in an increase of ubiquitin–protein conjugates of high molecular weight in epithelial cells after both 4 and 24 h, as expected. Interestingly, the specific 30 and 35 kDa ubiquitin–protein conjugates were not observed with epoxomicin alone after 4 h, indicating that they are specifically caused by CS exposure.

### Cigarette smoke activates the ER stress response marker eIF2 $\alpha$ in alveolar epithelial cells

We assume that CS-induced protein damage, as indicated by increased ubiquitination (Fig. 1), leads to the accumulation of damaged proteins inside the ER, hence inducing ER stress and subsequent UPR. One of the ER stress response markers is activated eIF2 $\alpha$ . We focus on this arm of UPR because it is the one directly involved in protein synthesis, which is important for normal protein homeostasis. Activation of eIF2 $\alpha$  occurs by phosphorylation at serine 51, which consequently leads to a transient repression of global protein synthesis. Figure 2 shows that exposure of A549 alveolar epithelial cells to CS and subsequent cultivation for 0.5 h produced a dose-dependent increase in the amount of phosphorylated eIF2 $\alpha$  (P-eIF2 $\alpha$ ). Dithiothreitol (an ER stress-inducing agent; 2 mM) was used as a positive control. Interestingly, a long-term (4 h) incubation of A549 cells with the proteasomal inhibitor epoxomicin (1  $\mu\text{M}$ ) also induced

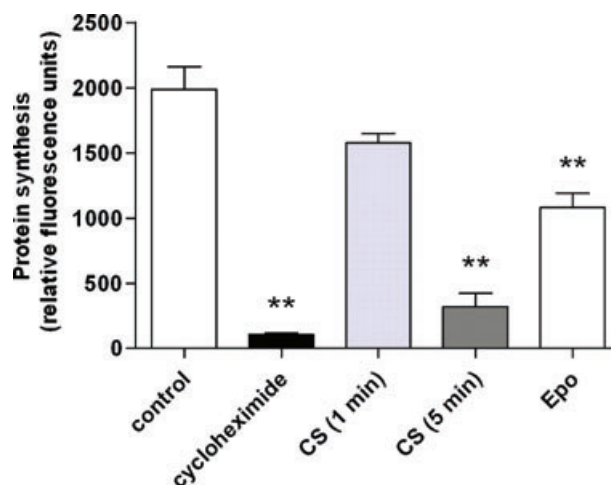


**Figure 2.** Cigarette smoke activates the endoplasmic reticulum (ER) stress response marker eIF2 $\alpha$  in alveolar epithelial cells. Western blot analysis of phosphorylated (P-eIF2 $\alpha$ ) and total eIF2 $\alpha$  in A549 cells exposed to CS (for 1 or 5 min) and cultured for 0.5 h or to epoxomicin (1  $\mu\text{M}$ ) and cultured for 4 h. Control cells were exposed to air (instead of CS) for 5 min (negative control). Dithiothreitol (DTT; 2 mM), an ER stress-inducing agent, was used as a positive control. The experiment was carried out using three independent replicates; a representative one is shown.

eIF2 $\alpha$  phosphorylation (Fig. 2), demonstrating that accumulation of ubiquitinated proteins (Fig. 1) leads to ER stress. However, epoxomicin caused a less intense ER stress response only after 4 h, while 5 min exposure of cells to CS induced a much stronger ER stress response after 0.5 h, also in comparison to the DTT positive control, despite forming slightly less ubiquitin–protein conjugates than epoxomicin. Results show the activation of this branch of the UPR in alveolar epithelial cells caused by CS.

### Cigarette smoke causes a decrease in nascent protein synthesis in alveolar epithelial cells

We tested the rate of nascent protein synthesis in cells exposed to CS and the proteasomal inhibitor epoxomicin, both of which are inducers of the ER stress response (as shown in Fig. 2). Cycloheximide, an inhibitor of protein synthesis, was used as a positive control. The analysis showed that *de novo* protein synthesis in A549 alveolar epithelial cells was decreased upon exposure to CS for 1 and 5 min [Fig. 3; CS (1 min),  $-410 \pm 73$  relative fluorescent units, non-significantly; and CS (5 min),  $-1667 \pm 105$  relative fluorescent units,  $P < 0.01$ ]. This indicates that CS has an immediate and strong inhibitory effect on the protein synthesis machinery, which can be explained as an attempt of the cell to re-establish protein homeostasis after exposure to CS and prior to starting the synthesis of new proteins. Epoxomicin also reduced nascent protein synthesis ( $-906 \pm 111$  relative



**Figure 3.** Cigarette smoke causes a decrease in protein synthesis in alveolar epithelial cells

Protein biosynthesis was analysed using flow cytometry after L-azidohomoalanine incorporation and fluorescence labelling ( $n = 4$ ). Cells were exposed to CS (for 1 or 5 min) or to epoxomicin (1  $\mu\text{M}$ ) and cultured for the following 4 h. Control cells were exposed to air (instead of CS) for 5 min. Cycloheximide, an inhibitor of protein synthesis, was used as a positive control. \*\* $P < 0.01$  versus control conditions.

fluorescent units,  $P < 0.01$ ), indicating that inhibition of the proteasome leads to the accumulation of aberrantly folded proteins in the ER, which cannot be degraded by the proteasome, and this in turn disturbs normal function of the ER and induces the UPR. In line with the stronger eIF2 $\alpha$  phosphorylation, CS showed a more powerful effect than epoxomicin on the reduction in protein synthesis.

### Cigarette smoke-induced increase in free amino acid levels requires proteasomal activity

Previously, we reported that CS induces an increase in free intracellular amino acid levels in alveolar epithelial cells (Abello *et al.* 2008). Here we wanted to relate an increase in free amino acids to proteasomal activities. We therefore exposed A549 cells to air (negative control), epoxomicin (positive control), CS or epoxomicin plus CS for 5 min and cultured the cells for another 4 h. Exposure to CS alone caused a significant increase in free amino acids in the lysates of A549 cells compared with air control conditions ( $335.7 \pm 87.9\%$ ,  $P < 0.05$ ; Fig. 4). Blocking the proteasomal activity with epoxomicin alone did not cause a significant change in free amino acid content. Interestingly, incubation with epoxomicin and subsequent exposure to CS resulted in a significant decrease in free amino acid levels inside A549 cells compared with CS-treated cells (change of  $-87.3 \pm 4.1\%$ ,  $P < 0.01$ ; Fig. 4). These results demonstrate that the CS-induced increase in

free amino acids appears to be a result of the increase in damaged proteins that are degraded by the proteasomes.

### Cigarette smoke inhibits proteasomal activities

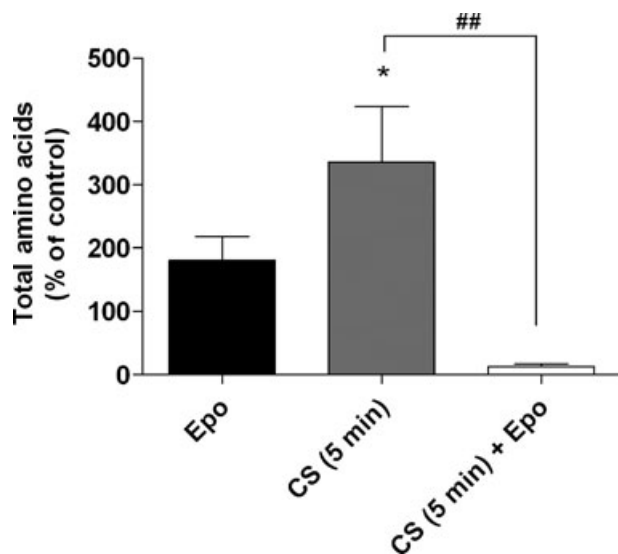
We examined the effect of CS on proteasomal activities to assess whether the proteasomes were still functional. We exposed A549 cells to CS for different time periods (1 or 5 min) and subsequently cultured them for another 4 or 24 h. All three proteasomal activities (caspase-, trypsin- and chymotrypsin-like activity) were measured using substrates presenting the corresponding cleavage sites (Fig. 5A–C). Epoxomicin (an inhibitor of proteasomal activity;  $1 \mu\text{M}$ ), was used as a positive control. Caspase-, trypsin- and chymotrypsin-like proteasomal activities were significantly decreased in A549 cells exposed for either 1 or 5 min to CS and cultured for the next 4 h [caspase-like activity (1 min),  $-10.2 \pm 14.1\%$ , non-significantly; caspase-like activity (5 min),  $-52.1 \pm 10.5\%$ ,  $P < 0.01$ ; trypsin-like activity (1 min),  $-24.7 \pm 5.1\%$ ,  $P < 0.01$ ; trypsin-like activity (5 min),  $-83.3 \pm 3.3\%$ ,  $P < 0.01$ ; chymotrypsin-like activity (1 min),  $-22.3 \pm 5.5\%$ ,  $P < 0.05$ ; and chymotrypsin-like activity (5 min),  $-61.3 \pm 9.2\%$ ,  $P < 0.01$ ]. A similar pattern was observed when leaving cells in culture for 24 h after CS exposure for either 1 or 5 min. These results demonstrate that exposure to CS reduces all three proteasomal activities in a dose-dependent manner.

### Cigarette smoke is cytotoxic to alveolar epithelial cells in culture

We exposed A549 cells to gas-phase CS to assess lactate dehydrogenase release as an indicator of cell membrane integrity (Fig. 6). Lactate dehydrogenase activity was significantly increased in the supernatant of A549 cells that were exposed to CS for 5 min and subsequently cultured for another 24 h. Exposure to CS for 1 min resulted in no significant increase in cytotoxicity in comparison to the control cells.

### Discussion

Cigarette smoke is the major aetiological factor for the development of COPD; however, the mechanisms of pathogenesis are still largely unclear. In the present study we investigated the effects of CS on protein turnover in a human alveolar epithelial cell line. Exposure to CS induces protein damage, as demonstrated by elevated levels of ubiquitin–protein conjugates, and triggers ER stress and subsequent UPR, indicated by activation of the ER stress response marker eIF2 $\alpha$  and subsequent significant reduction in nascent protein synthesis, accompanied



**Figure 4. Cigarette smoke increases free amino acid levels in alveolar epithelial cells**

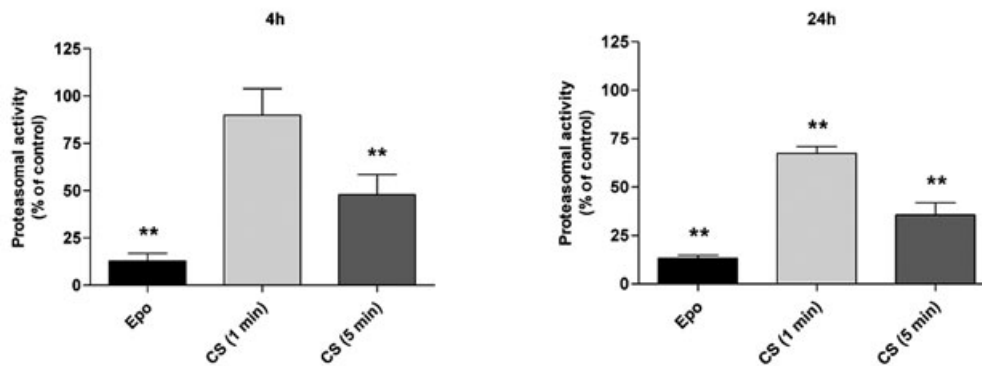
Free amino acids from A549 alveolar epithelial cells, exposed to air (as a negative control), Epo, CS for 5 min or epoxomicin plus CS for 5 min and cultured for 4 h, were quantified by liquid chromatography–mass spectrometry using stable isotope labelling ( $n = 3$ ). \* $P < 0.05$  versus control conditions, ## $P < 0.01$  versus CS (5 min).

by increased levels of free intracellular amino acids. Although UPR is normally associated with increased proteasomal activity to degrade damaged proteins, we observed a rapid and prolonged significant reduction of all three proteasomal activities (caspase-, trypsin- and chymotrypsin-like activity). Taken together, these findings indicate an overall disturbance in protein turnover inside

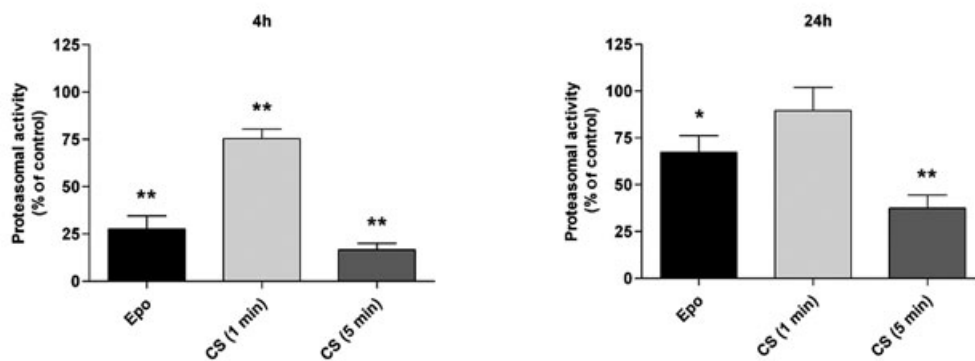
alveolar epithelial cells exposed to CS due to both the CS-induced ER stress response and proteasomal dysfunction.

Cigarette smoke, containing numerous oxidants and free radicals (Church & Pryor, 1985; Pryor & Stone, 1993), is capable of inducing oxidative modifications of cellular proteins. Damaged proteins are targeted for proteasomal degradation by conjugation with ubiquitin molecules

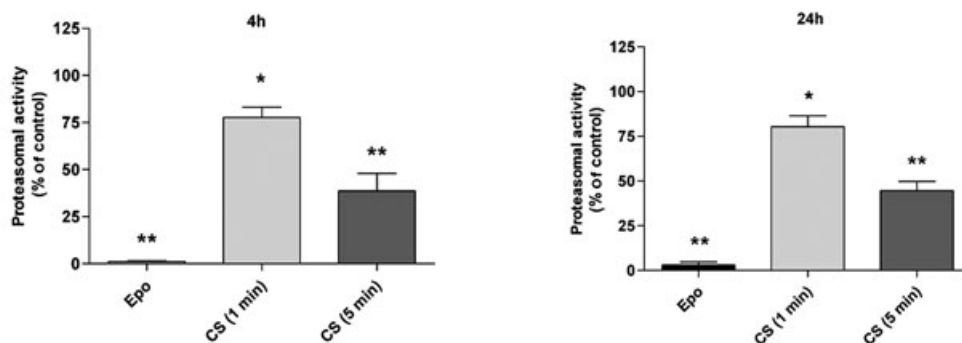
### A Caspase-like activity



### B Trypsin-like activity



### C Chymotrypsin-like activity



**Figure 5. Cigarette smoke disturbs proteasomal activities in alveolar epithelial cells**

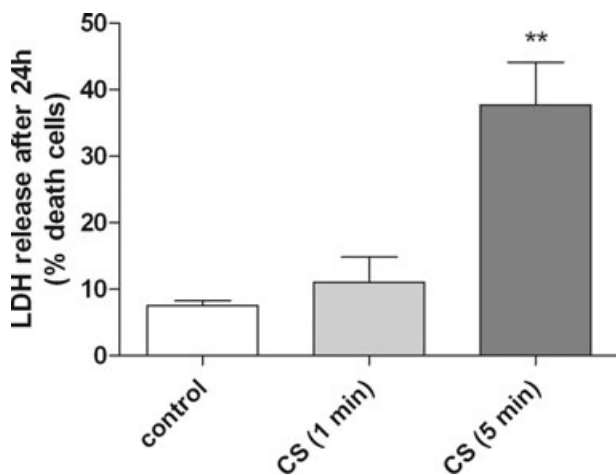
Three different fluorogenic substrates were used for proteasomal activities analysis ( $n = 4$ ). Control cells were exposed to air (instead of CS) for 5 min. Epoxomicin, an inhibitor of proteasomal activity, was used as a positive control. Caspase-like proteasomal activity (A), trypsin-like proteasomal activity (B) and chymotrypsin-like proteasomal activity (C) were measured in A549 cells exposed to CS for 1 or 5 min and cultured for the following 4 or 24 h. \* $P < 0.05$ , \*\* $P < 0.01$  versus control conditions.

(Hershko & Ciechanover, 1998; Sherman & Goldberg, 2001; Glickman & Ciechanover, 2002). Recently, Min *et al.* (2011) showed an increase in accumulation and aggregation of ubiquitinated proteins in lung tissue samples of COPD patients, dependent on the severity of emphysema; however, no relation to the patients' smoking history was shown. In our *in vitro* study we observed an increase in the level of ubiquitin–protein conjugates (with molecular masses around 30 and 35 kDa, as well as those above 150 kDa) in lysates of alveolar epithelial cells treated with gas-phase CS. Ottenheim *et al.* (2005) found similar changes in protein ubiquitination in diaphragm muscle homogenates from COPD patients. They reported additional ubiquitin–protein conjugates with molecular masses between 75 and 250 kDa and increased levels of those around 30 and 70 kDa. Furthermore, we found that the majority of ubiquitinated proteins were degraded during continued cell culture for 24 h. However, it remains unclear why some ubiquitin–protein conjugates (with molecular masses around 30 and 35 kDa) remained intact 24 h after CS exposure. A possible explanation for this observation could be that cellular stress caused by CS changed these specific proteins structurally in such a way that the proteasome was unable to recognize them as substrates tagged for proteolysis or that the proteasome was unable to unfold them. It would be interesting to reveal which proteins escaped degradation by the proteasome. Unfortunately, the use of gel electrophoresis for separation of proteins and immunoblotting with anti-ubiquitin antibodies is not sufficiently informative, because ubiquitin chains on proteins can vary greatly in length. Using more sophisticated proteomics methods in

future experiments might yield more information about the identity of these specific proteins. We assume that accumulation of such ubiquitinated proteins in the cytosol could lead to the loss of vital cellular functions, eventually causing cell death and triggering chronic inflammation in the lungs of smokers, possibly contributing to the pathogenesis of COPD (Min *et al.* 2011).

Upon exposure of cells to different stressors, accumulation of misfolded or unfolded proteins occurs inside the ER, leading to persistent ER stress and subsequent cell death (Haynes *et al.* 2004; Shore *et al.* 2011; Walter & Ron, 2011). In order to re-establish protein homeostasis, inhibition of protein translation may be promoted by the pancreatic ER kinase branch of the UPR (Todd *et al.* 2008). Pancreatic ER kinase activates eIF2 $\alpha$  by phosphorylation at serine 51, leading to a transient repression of global protein synthesis by preventing formation of the 80S initiation complex (Jiang & Wek, 2005; Jorgensen *et al.* 2008). Recent studies have shown that CS causes ER stress and induces UPR, both in cell culture (Hengstermann & Muller, 2008; Jorgensen *et al.* 2008; Kelsen *et al.* 2008; Tagawa *et al.* 2008; Geraghty *et al.* 2011) and in animal models (Malhotra *et al.* 2009; Geraghty *et al.* 2011), as well as in the lungs of smokers (Kelsen *et al.* 2008) and COPD patients (Min *et al.* 2011). Both Hengstermann & Muller (2008) and Geraghty *et al.* (2011) concluded that CS-induced ER stress primarily activates the pancreatic ER kinase pathway of the UPR. In the present study we show that gas-phase CS increases the amount of phosphorylated eIF2 $\alpha$  in a dose-dependent manner and decreases *de novo* protein synthesis in A549 alveolar epithelial cells. Reduced protein synthesis was also observed in the lungs of rats exposed to CS (Garrett & Jackson, 1980), patients with  $\alpha_1$ -antitrypsin deficiency-related emphysema (Golpon *et al.* 2004) and SA 3T3 mouse fibroblast cells exposed to aqueous extracts of CS (Hengstermann & Muller, 2008); however, this is the first study to show a direct effect of gas-phase CS on protein synthesis in human alveolar epithelial cells. Our results indicate that CS triggers a mechanism that inhibits protein translation, possibly allowing additional time for the cell to recover from CS-induced protein damage. To recover from defects caused by CS, alveolar epithelial cells must be able to migrate, proliferate and differentiate (Rennard, 1999); therefore, we suggest that prolonged inhibition of protein synthesis in alveolar epithelial cells caused by CS may contribute to the ineffective repair and may result in altered alveolar structures, as observed in the lungs of COPD patients (Rennard, 1999).

In line with our previous studies (Abello *et al.* 2008), we demonstrate that the intracellular level of free amino acids greatly increases upon exposure of alveolar epithelial cells to gas-phase CS. In addition, we show that despite the substantial inhibition of proteasomal activities by CS exposure the increase in free amino acids is mediated



**Figure 6.** Cigarette smoke is cytotoxic to alveolar epithelial cells. A549 alveolar epithelial cells were exposed to air (as a control) or CS (for 1 or 5 min) and cultured for another 24 h ( $n = 3$ ). Following the incubation period, the activity of lactate dehydrogenase (LDH) released into the cell culture medium was measured. \*\* $P < 0.01$  versus control.



by proteasomal degradation, because it was completely blocked by epoxomicin. Thus, exposure to CS increases the level of ubiquitin–protein conjugates in epithelial cells, leading to protein degradation by the proteasome followed by further degradation of the resulting peptides into free amino acids. All in all, the increase in most free amino acids can be interpreted as a result of enhanced protein degradation by the proteasome together with a reduction in nascent protein synthesis upon exposure to CS.

Beside the inhibition of protein translation, the UPR is trying to restore protein homeostasis by facilitating damaged protein degradation through the ubiquitin–proteasome pathway. It has been shown that moderately oxidized proteins are rapidly degraded by the ubiquitin–proteasome pathway, but severely oxidized, cross-linked proteins are poor substrates for degradation and tend to accumulate in the cell, probably in aggregates, leading to inhibition of the ubiquitin–proteasome system (Bence *et al.* 2001; Davies, 2001; Shringarpure & Davies, 2002); however, there is no direct evidence concerning the effect of CS on the ubiquitin–proteasome pathway. Recently, Malhotra *et al.* (2009) demonstrated a marked decrease in total proteasomal activity caused by impaired Nrf2 (an anti-oxidant transcription factor) signalling in the lungs of patients with mild and advanced COPD, implying its role in COPD progression. Our results with alveolar epithelial cells exposed to gas-phase CS show that proteasomal activities are significantly decreased and that the effect is dose and time dependent. We therefore speculate that the observed CS-induced inhibition of proteasomal activities is mediated by accumulation of severely oxidized proteins produced by CS. Another possible mechanism underlying proteasomal inhibition could be that subunits of the proteasome are directly oxidatively modified by ROS generated by CS and therefore the proteasome is less active towards its substrates. Reinheckel *et al.* (2000) suggested that the high susceptibility of the 26S proteasome in human haematopoietic K562 cells exposed to H<sub>2</sub>O<sub>2</sub> is due to oxidation of essential amino acids in the proteasome activator PA 700, which mediates ATP-dependent proteolysis. Previously, the same group reported that the 26S proteasome is rather susceptible to oxidative stress, whereas the 20S proteasome is more resistant (Reinheckel *et al.* 1998). Proteolysis by the 26S proteasome requires not only polyubiquitin recognition, but also ATP hydrolysis (Glickman & Ciechanover, 2002). In our previous work, we showed that CS disturbed mitochondrial function and thereby decreased ATP synthesis (van der Toorn *et al.* 2007a). These facts give support to a new hypothesis stating that not only the structural changes of proteasomal subunits, but also the intracellular ATP depletion caused by CS might contribute to a decrease in proteasomal activities. Finally, reduction in proteasomal activity by CS leads to aberrant accumulation of ubiquitinated substrates, making this

branch of the UPR insufficiently effective to re-establish cellular homeostasis. An ineffective repair process, which is part of the inflammatory response to CS-induced injury of lung structures, may then result in the altered alveolar structure and tissue dysfunction that is characteristic of COPD (Rennard, 1999).

In conclusion, our data support the hypothesis that CS severely disturbs protein homeostasis inside alveolar epithelial cells by inducing protein damage and an ER stress response. Activation of the UPR leads to reduced nascent protein synthesis; however, proteasomal activities are significantly disturbed by CS, making the ubiquitin–proteasome system ineffective to remove all the damaged proteins, which leads to the accumulation of damaged proteins and a sustained UPR. Prolonged ER stress may lead to excessive cell death with disruption of the epithelial barrier, contributing to the development of COPD.

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