Airway epithelial innate host defence in chronic obstructive pulmonary disease

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CHAPTER 7

Effect of acute cigarette smoke exposure on airway epithelial activation of the integrated stress response.

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ABSTRACT

Maintaining airway epithelial integrity is critical for lung defense. However, exposure to inhaled micro-organisms and toxicants constitutes a continuous threat to this defense. The integrated stress response (ISR) is a cellular mechanism that may contribute to the protection and recovery of airway epithelial cells upon injury. However, especially following chronic activation, the ISR may also mediate cell death. It has been proposed that the ISR contributes to the development and/or progression of the lung disorder chronic obstructive pulmonary disease (COPD). Moreover, it has been shown that prolonged exposure of cultured airway epithelial cells to cigarette smoke, which is the main risk factor of COPD, causes ISR-mediated cell death. This suggests a role of the ISR in smoke-induced effects on the airway epithelium that may contribute to COPD development. However, activation of the ISR has been investigated mainly in cell lines, while activation in primary airway epithelial cell cultures from COPD patients has not been studied. In the present study, we therefore further examined the influence of acute exposure to whole cigarette smoke or cigarette smoke extract on activation of the ISR in differentiated and undifferentiated airway epithelial cells respectively. We demonstrate that the ISR is activated in both smoke exposure models. Moreover, we observed higher expression of ISR-related target genes in whole smoke-exposed COPD cell cultures compared to controls, and provide data to propose a mechanism by which smoke-induced oxidative stress modulates the ISR. Taken together, our findings provide evidence for the involvement of activation of the ISR in the airway epithelial response to cigarette smoking and the importance of oxidative stress in modulating this response. More pronounced activation of the ISR in smoke-exposed epithelial cells from COPD patients, suggests involvement of the ISR in COPD pathogenesis.

INTRODUCTION

The airway epithelium is the first cellular defense lining of the respiratory tract and provides protection through its barrier function, mucociliary clearance, and innate immune defense mechanisms (1-4). Environmental stressors, including inhaled pathogens and cytotoxic particles and gases, may have detrimental effects on the airway epithelium, causing injury and promoting cell death (5). Adaptation of airway epithelial cells to stressors is therefore critical, and various intrinsic mechanisms provide protection from inhaled toxicants, including the detoxification by metabolic enzymes and antioxidants (6).

The main risk factor for the development and progression of the inflammatory lung disorder chronic obstructive pulmonary disease (COPD) in Westernized societies is cigarette smoking (7). Various studies have provided evidence that cigarette smoke exposure results in activation of the unfolded protein response (UPR) to endoplasmic reticulum (ER) stress (8-11). It was shown that cigarette smoke extract may selectively activate the PERK arm of the UPR in the bronchial epithelial cell line BEAS-2B and that this could be inhibited by anti-oxidants (12). PERK is an ER stress-sensing kinase that phosphorylates the eukaryotic translation initiation factor 2 (eIF2) subunit, eIF2α (13). Since eIF2α can also be phosphorylated by other stress-sensing kinases (GCN2, HRI and PKR), the pathway starting with phosphorylation of eIF2α is also referred to as the integrated stress response (ISR) (14). Phosphorylation and inactivation of eIF2α leads to the protection of the cell by inducing a broad inhibition of protein translation. This inhibition may provide protection against e.g. the accumulation of misfolded proteins in the ER. Phosphorylation of eIF2α also results in activation of gene expression through selective translation of the transcription factor ATF4, resulting in production of the transcription factor C/EBP homologous protein (CHOP), that regulates expression of a range of genes including growth arrest and DNA damage-inducible protein 34 (GADD34) (15). Interestingly, the gene expression signature present in bronchial tissue from COPD patients is enriched for genes regulated by ATF4 (16). By dephosphorylating eIF2α, GADD34 enables the recovery of protein synthesis and thus controls the duration of ISR. In contrast, during sustained stress, recovery of protein translation by GADD34 may promote cell death (15). GADD34 is therefore a critical regulator of cell viability, which depends on the resolution or persistence of cellular stress.

Although evidence suggests that the UPR and ISR can be activated by cigarette smoke (CS), many studies were performed in cell lines or undifferentiated primary cells using CS extract. Consequently, relatively little is known about activation of the UPR and ISR in differentiated primary airway epithelial cells by whole CS, or whether these responses are affected by the COPD status of the tissue donor. This is important, since we and others have shown that biochemical features of COPD may persist in epithelial cell cultures *ex vivo* (17). Moreover, the mechanistic relationship between CS and activation of the ISR remains obscure.

In a previous study we showed that short-term exposure of airway epithelial cells to whole CS had a mild cytotoxic effect, while cells displayed transient inflammatory responses and a temporary impairment in the epithelial barrier integrity (18). Activation of the ISR might contribute to this recovery of epithelial cells after acute CS exposure. Therefore, in the present study we examined ISR activation in airway epithelial cells during short-term exposures

to whole CS, and compared the activation of the ISR between cell cultures derived from individuals with COPD and non-COPD controls. We provide evidence that CS increases expression of GADD34 both via the classical ISR and via a non-ISR oxidative stress-dependent pathway. These findings suggest a new mechanism by which oxidative stress can modulate activation of the ISR.

MATERIALS AND METHODS

Cell culture

Primary bronchial epithelial cells (PBEC) were isolated from tumour-free resected lung tissue obtained during surgery for lung cancer, and cultured at the air-liquid interface (ALI-) or submerged conditions (S-). Culturing of ALI-PBEC and S-PBEC was performed as previously reported (18). In ALI-PBEC cultures, cells were seeded on 0.4 μm pore sized semi-permeable transwell membranes (Corning Costar, Cambridge, MA, USA) and first cultured in submerged conditions in a 1:1 mixture of bronchial epithelial growth medium (BEGM) (Lonza, Verviers, Belgium) and Dulbecco's modified Eagle's medium (DMEM) (Gibco, Bleiswijk, The Netherlands) containing 1 mM HEPES (Lonza), 100 U/mL penicillin and 100 μg/ml streptomycin (Lonza) (hereafter referred to as B/D culture medium), supplemented with singleQuot BEGM supplements (except Gentamycin) (Lonza), 15 ng/ml retinoic acid (Sigma-Aldrich) and 1 mg/ml BSA (Sigma-Aldrich). When monolayers were confluent, the apical medium was removed and cells were cultured in air-exposed conditions for at least 2 weeks to allow mucociliary differentiation. In western blot experiments, cells were cultured overnight and during the experiment with starvation medium, consisting of B/D medium without BSA and the SingleQuot supplements BPE and EGF. ALI-PBEC cultures were used from COPD patients and non-COPD (ex)smokers, for whom the disease status was determined based on pre-surgery lung function data according to the Global Initiative for Chronic Obstructive Lung Disease classification (7) (Table 1). S-PBEC were cultured in regular tissue culture plates with B/D culture medium as earlier described, but without the additional 15 ng/ml retinoic acid supplementation. Cells were used when approximately 80-90% confluent. In all experiments, S-PBEC were cultured overnight prior to the experiment and during the experiment with starvation medium, which was similar to ALI-PBEC starvation medium, but lacked the additional 15 ng/ml retinoic acid supplementation. eIF2α^{AA} and eIF2α^{SS} mouse embryonic fibroblasts (MEFs) were cultured as previously described (19).

Cigarette smoke exposure models and other stimuli

ALI-PBEC cultures were exposed to whole cigarette smoke (CS) using an exposure model previously described (18). In short, cells were exposed to whole smoke derived from one standard research cigarette (3R4F), after which cells were incubated for the indicated periods of time. S-PBEC and MEF cultures were treated with cigarette smoke condensate (CSC), which was prepared as previously reported (20). Cells were treated with CSC via a "pulse" method described in Figure 4 and corresponding text in the Results section, similar to the whole CS exposure protocol. In indicated experiments, cells were pre-treated for 1 hour and treated during the experiment with the anti-oxidant N-acetylcysteine (NAC) (Sigma). As indicated, in some experiments cells were treated with thapsigargin (Tg) or tunicamycin (Tm) (both Sigma) as positive controls for ER stress

Table 1: Characteristics of COPD and non-COPD patients.

	COPD	non-COPD	p-value
Number of donors	7	6	
Gender (females/males)	2/5	2/4	
Age, years	58±7	67±9	
FEV_1, % predicted	64±11	96±20	< 0.002
FEV_1/FVC %	53±8	76±4	< 0.0001

Patient characteristics: Age and lung function are shown as means ± SD. The mean differences in FEV_1 (% predicted) and FEV_1/FVC (%) were compared using the non-parametric Mann-Whitney test.

Western blot

Cell lysates were prepared in Harvest Buffer (Buffer H), consisting of 10 mM HEPES pH 7.9, 50 mM NaCl, 0.5 mM sucrose, 0.1 mM EDTA, 0.5% (v/v) Triton X-100, 1 mM DTT, Protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany), phosphatase inhibitors (10 mM tetrasodium pyrophosphate, 17.5 mM β-glycerophosphate, and 100 mM NaF), and 1 mM PMSF. Nuclear extraction was performed using the NE-PER Nuclear Protein Extraction Kit (Thermo Scientific, Rockford, IL) according to the manufacturer's protocol. Cell lysates were diluted in 4x sample buffer consisting of 0.2 M Tris-HCl pH 6.8, 16% [v/v] glycerol, 4% [w/v] SDS, 4% [v/v] 2-mercaptoethanol and 0.003% [w/v] bromophenol blue. Samples were separated using a 10% SDS-PAGE gel and proteins were transferred onto nitrocellulose membrane. Primary antibodies were used against: eIF2α, phospho-eIF2α, ATF4, TBP (Cell Signaling Technology), GADD34 (ProteinTech) and puromycin (Millipore) (all 1:1000 diluted). After secondary antibody incubation, proteins detection was visualized using ECL (ThermoScientific) or the LI-COR Odyssey Infrared Imaging System (LI-COR Biosciences). Quantification of protein bands was done by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

qPCR

RNA was isolated with the Maxwell tissue RNA extraction kit (Promega) or Qiagen RNeasy mini kit (Qiagen). cDNA synthesis and qPCR was performed as previously described (see Table 2 for qPCR primer pairs) (18, 19). Relative gene expression was calculated according to the standard curve method. ATP5B and RPL13A were selected using the "Genorm method" (Genorm; Primer Design, Southampton, UK) as reference genes for PBEC. β-actin was used as reference gene for MEFs.

ELISA & LDH release assay

Protein secretion of IL-8/CXCL8 (R&D systems), and LDH release (Roche) was determined in the cell culture medium according to the manufacturer's protocol.

Statistics

Results were analyzed using GraphPad Prism 6.0 (GraphPad Inc). As indicated in the figures, statistical tests used for data analysis were (un)paired t-test or 2-way ANOVA, with post-hoc Bonferroni correction for multiple analyses. Differences with a p-value < 0.05 were considered statistically significant.

Table 2 qPCR primers

Gene	Forward primer (5' - 3')	Reverse primer (5' - 3')
Human		
GADD34	ATGTATGGTGAGCGAGAGGC	GCAGTGTCCTTATCAGAAGGC
CHOP	GCACCTCCCAGAGCCCTCACTCTCC	GTCTACTCCAAGCCTTCCCCCTGCG
Bip/GRP78	CGAGGAGGAGGACAAGAAGG	CACCTTGAACGGCAAGAACT
sXBP1	TGCTGAGTCCGCAGCAGGTG	GCTGGCAGGCTCTGGGGAAG
IL8	CAGCCTTCCTGATTTCTG	CACTTCTCCACAACCCTCTGC
RPL13A	AAGGTGGTGGTCGTACGCTGTG	CGGGAAGGGTTGGTGTTCATCC
ATP5B	TCACCCAGGCTGGTTCAGA	AGTGGCCAGGGTAGGCTGAT
PTGS2	TAAGTGCGATTGTACCCGGAC	TTTGTAGCCATAGTCAGCATTGT
DUSP5	TGTCGTCCTCACCTCGCTA	GGGCTCTCTCACTCTCAATCTTC
cFOS	CCTAACCGCCACGATGATGT	TCTGCGGGTGAGTGGTAGTA
Mouse		
GADD34	CCCGAGATTCCTCTAAAAGC	CCAGACAGCAAGGAAATGG
CHOP	GGAGCTGGAAGCCTGGTATGAG	GCAGGGTCAAGAGTAGTGAAGG
ACTB	TCCTGGCCTCACTGTCCA	GTCCGCCTAGAAGCACTTGC

RESULTS

Whole cigarette smoke exposure increases the ISR in ALI-PBEC

We first examined the effect of whole cigarette smoke (CS) on activation of the integrated stress response (ISR) in mucociliary differentiated primary bronchial epithelial cells (ALI-PBEC). Cell cultures were exposed to whole CS from a single cigarette (Figure 1A), which in previous studies was shown to cause an acute and transient disruption of the epithelial barrier, impairment of wound repair and induction of innate immune responses with minimal cytotoxic effects (17, 18, 21). An acute activation of the ISR upon CS exposure was detected based on the increased phosphorylation of eIF2α and nuclear accumulation of ATF4 within the first 2 hours after exposure (Figure 1B,C). In addition, a time-dependent expression of *GADD34* and *CHOP* mRNA was observed, which continued to increase up to 2 hours after exposure (Figure 1D). In previous studies it has been shown that smoke-induced activation of the ISR pathway is induced by the unfolded protein response (UPR) to endoplasmic reticulum (ER) stress (12). Therefore, we further examined activation of the UPR by determining the expression of the UPR target gene *BIP/GPR78* and splicing of *XBP1* mRNA. We did not observe an increased expression of *BIP* nor increased splicing of *XBP1* mRNA within the 2 h timeframe that was examined (Figure 1E). Similar results were obtained when expression levels of *BIP* and spliced *XBP1* were determined at 6 h after CS exposure, by which time the ER stress inducer tunicamycin was able to increase the expression of both markers in ALI-PBEC (Figure 1F). In contrast, both the levels of *CHOP* and *GADD34* mRNA expression were induced by both CS and tunicamyin at 6 hours.

The persistence of CS-induced ISR activation was subsequently determined at time points ranging from 0-24 h post-CS exposure (Figure 2A) by determining GADD34 protein

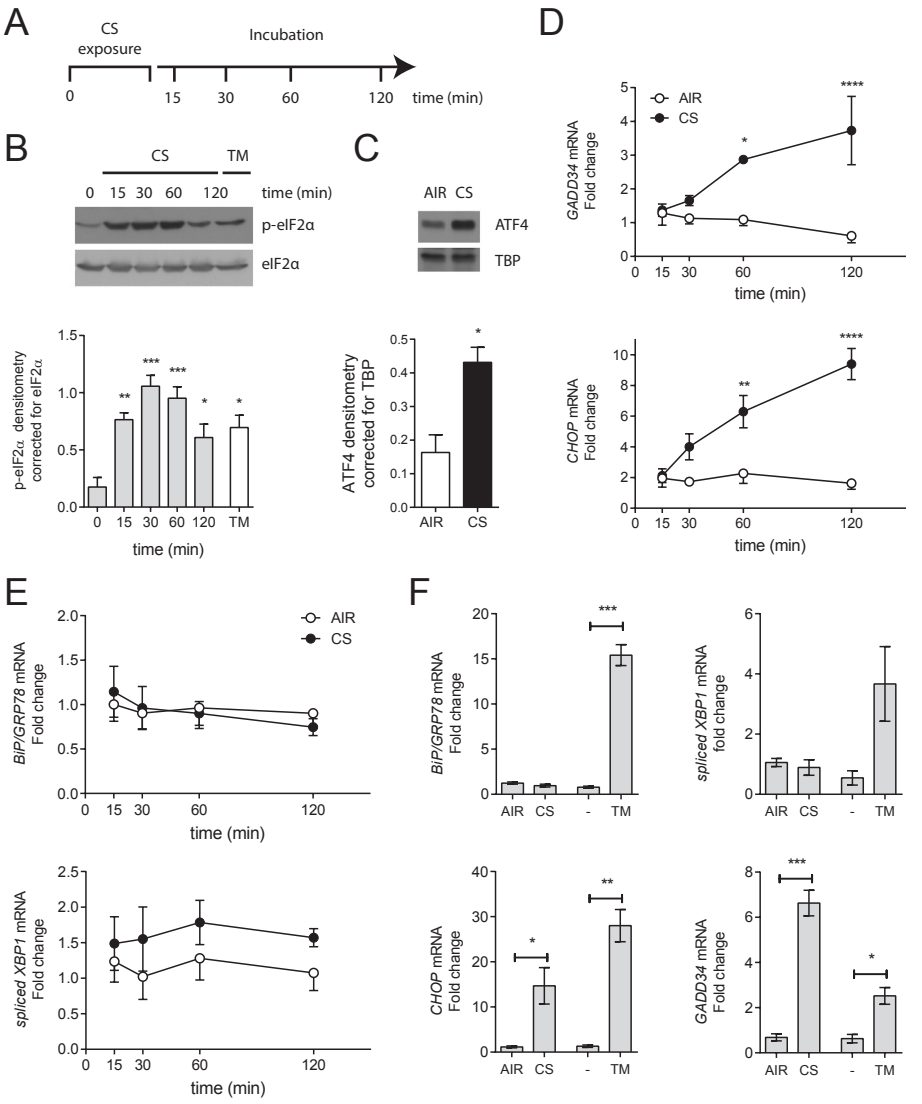

Figure 1. Effect of whole cigarette smoke (CS) exposure on ISR-activation in ALI-PBEC cultures. (A) Schematic of the whole CS exposure experiment, in which ALI-PBEC were exposed to CS from a single cigarette, followed by incubation for different time periods ranging from 15-120 min. (B) eIF2α phosphorylation was determined 15-120 min after whole CS exposure. The ER stress inducing agent tunicamycin (TM) was used as positive control. Results were quantified by densitometry using total eIF2α as loading control. (C) Nuclear localization of ATF4 was determined 2 h after CS exposure. Results were quantified by densitometry using TATA binding protein (TBP) as loading control. (D) Analysis of *GADD34* and *CHOP*, (E) BiP/*GRP78* and *sXBP1* mRNA expression 15-120 min after CS exposure. (F) mRNA expression of *GADD34*, *CHOP*, BiP/*GRP78* and *sXBP1* after Air/CS exposure or TM after 6 h incubation period. Results are shown as mean ± SD of at least 3 different donors. Analysis of differences was conducted using (B,D,E) a one-way ANOVA with a Bonferroni post-hoc test and (C,F) a paired t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0005$.

expression, eIF2α phosphorylation, and puromycin incorporation as measurement of protein synthesis. Increased GADD34 protein expression was observed at 3 h and peaked at 6 h after CS exposure (Figure 2B,C). This was preceded by an earlier increase in CS-induced eIF2α phosphorylation (Figure 1B), which fell below baseline levels at 6-12 h before returning to baseline by 24 h (Figure 2B,C). CS exposure impaired protein synthesis, as reported by puromycin incorporation, and this inhibition was most prominent at 3 h after CS exposure but protein synthesis progressively recovered at 6, 12 and 24 h after exposure. Taken together, these findings suggest that acute exposure to whole CS causes a transient activation of the ISR.

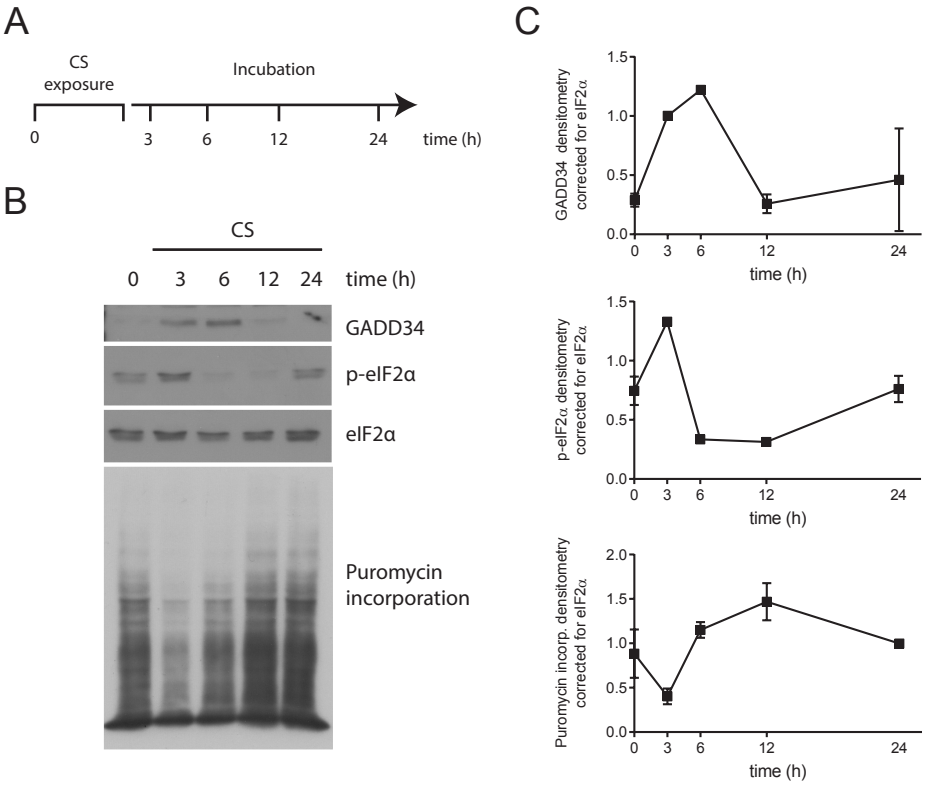

Figure 2. Effect of whole CS exposure on protein translation in ALI-PBEC cultures. (A) Schematic of the experiment, in which ALI-PBEC were exposed to CS from a single cigarette, followed by incubation for different time periods ranging from 0-24 h. (B) Analysis of GADD34 protein expression, eIF2α phosphorylation, and puromycin incorporation as marker of protein translation. (C) Results were quantified by densitometry using total eIF2α as loading control. Experiments were conducted in 2 independent donors showing similar results.

COPD airway epithelial cells display higher CS-induced expression of CHOP and GADD34

Next, we examined the induction of the ISR in ALI-PBEC from COPD patients and non-COPD (ex)smokers by comparing expression of *CHOP* and *GADD34* mRNA. ALI-PBEC were exposed to whole CS and mRNA expression levels were determined at 3, 12 and 24 h after exposure. In line with earlier observations, CS-induced *CHOP* and *GADD34* expression was observed early after exposure, whereas the expression decreased at later time points (Figure

3A). A comparison between COPD and non-COPD airway epithelial responses demonstrated a significantly higher expression of *GADD34* and *CHOP* in COPD airway epithelial cultures. In addition, and in line with this observation, we observed a negative correlation between CS-induced GADD34 and CHOP expression and FEV_1 (Figure 3B) and FEV_1/FVC (Figure 3C).

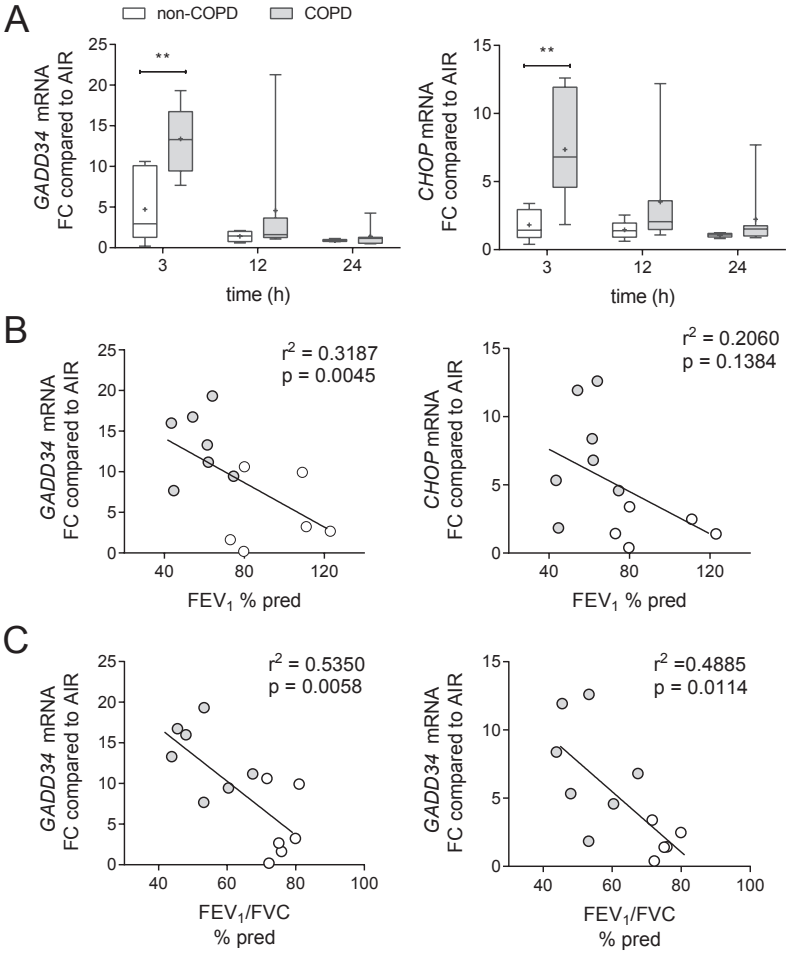

Figure 3. CS-induced expression of GADD34 and CHOP in COPD and non-COPD ALI-PBEC. (A) ALI-PBEC from COPD and non-COPD donors were exposed to whole CS and incubated for 3,12, and 24 h after which *GADD34* and *CHOP* mRNA expression was determined. (B) Correlation between fold change in mRNA expression compared to air controls of *GADD34* and *CHOP* mRNA, with FEV_1 (% pred), and (C) FEV_1/FVC (% pred). n=7 COPD and n=6 non-COPD donors were used in the analysis. Analysis of differences was conducted using a one-way ANOVA with a Bonferroni *post-hoc* test. ** $p < 0.01$. Correlations were assessed by determining the linear regression.

In addition to *GADD34* and *CHOP*, we also determined the expression of *PTGS2*, *DUSP5* and *cFOS,* which have previously been found to be enhanced in airway epithelial tissue from COPD patients and to be linked with ATF4-dependent gene transcription (16). However,

whereas expression of these genes was also transiently increased at 3 hours post-CS exposure, we did not observe differences in the expression of these genes between COPD patients and non-COPD controls (Supplementary Figure 1).

Cigarette smoke extract increases the ISR in submerged cultured PBEC

Although whole CS exposure of ALI-cultured PBEC may adequately mimic *in vivo* smoke exposure of epithelial cells in lung tissue, this model does not readily allow exploration of the mechanisms regulating ISR activation. Therefore we also determined the effect of short-term cigarette smoke exposure on ISR activation using cigarette smoke condensate (CSC), which can be used in conventional submerged cell cultures. First, we examined the effect of CSC on submerged cultured primary bronchial epithelial cells (S-PBEC), to determine whether activation of the ISR could be reproduced in this model. Similar to ALI-PBEC exposed to whole CS, S-PBEC were stimulated using a pulse exposure method in which cells were incubated with CSC for 15 minutes, after which the culture medium was refreshed and cells were incubated for different time periods (Figure 4A). This was done using CSC concentrations that in a previous study were shown to be cytotoxic to epithelial cells upon prolonged exposure (20). In contrast to prolonged exposures, pulse exposure only caused a mild cytotoxic effect based on the release of LDH in the culture medium (Figure 4B). However, CSC exposure of S-PBEC did cause a dose-dependent protein secretion of the neutrophil chemoattractant IL-8/CXCL8 (Figure 4C). This was accompanied by an early and dose-dependent mRNA expression of *IL8* (Figure 4D,E). These findings suggest that, similar to our studies using the whole CS exposure model (18), pulsed CSC stimulation also induces pro-inflammatory responses with minimal cytotoxic effects. We next determined ISR activation in S-PBEC stimulated with CSC by determining mRNA expression of *CHOP* and *GADD34*. Similar to *IL8*, *GADD34* and *CHOP* mRNA expression was rapidly but transiently induced, reaching maximal levels at 3 h after exposure (Figure 4D). Moreover, a dose dependent increase of *GADD34* and *CHOP* mRNA expression was observed at 3 h after exposure (Figure 4E). Interestingly, in contrast to our observations with differentiated ALI-PBEC, CSC also increased mRNA expression of *BIP* and spliced *XBP1*, peaking at 6 h after exposure (Figure 4F).

CSC –induced GADD34 expression occurs independent of the ISR

Next, to gain insight into the mechanism by which CS induced expression of *GADD34* and *CHOP*, we repeated the exposure to CSC experiments in MEFs, comparing wild type eIF2α^{SS} cells with MEFs lacking the ability to phosphorylate eIF2α owing to mutation of serine-51, eIF2α^{AA} (22). As expected, CSC induced expression of both *CHOP* and *GADD34* in eIF2α^{SS} MEFs, however, in eIF2α^{AA} MEFs CSC remained able to induce GADD34 expression, but the induction of CHOP was lost (Figure 5A). Indeed, in eIF2α^{AA} MEFs CSC induced GADD34 at a concentration below that required to induce GADD34 in wild type cells. In line with this observation, increased levels of GADD34 protein was also observed in both eIF2α^{AA} and eIF2α^{SS} MEFs following (Figure 5B), suggesting that an ISR-independent mechanism contributes to CSC-induced GADD34 expression.

ISR-deficient cells are known to be impaired in their response to oxidative stress (23). It is also known that altered redox plays an important role in the cytotoxic effects of cigarette smoke (20). We therefore determined whether the ISR-independent expression of GADD34 might

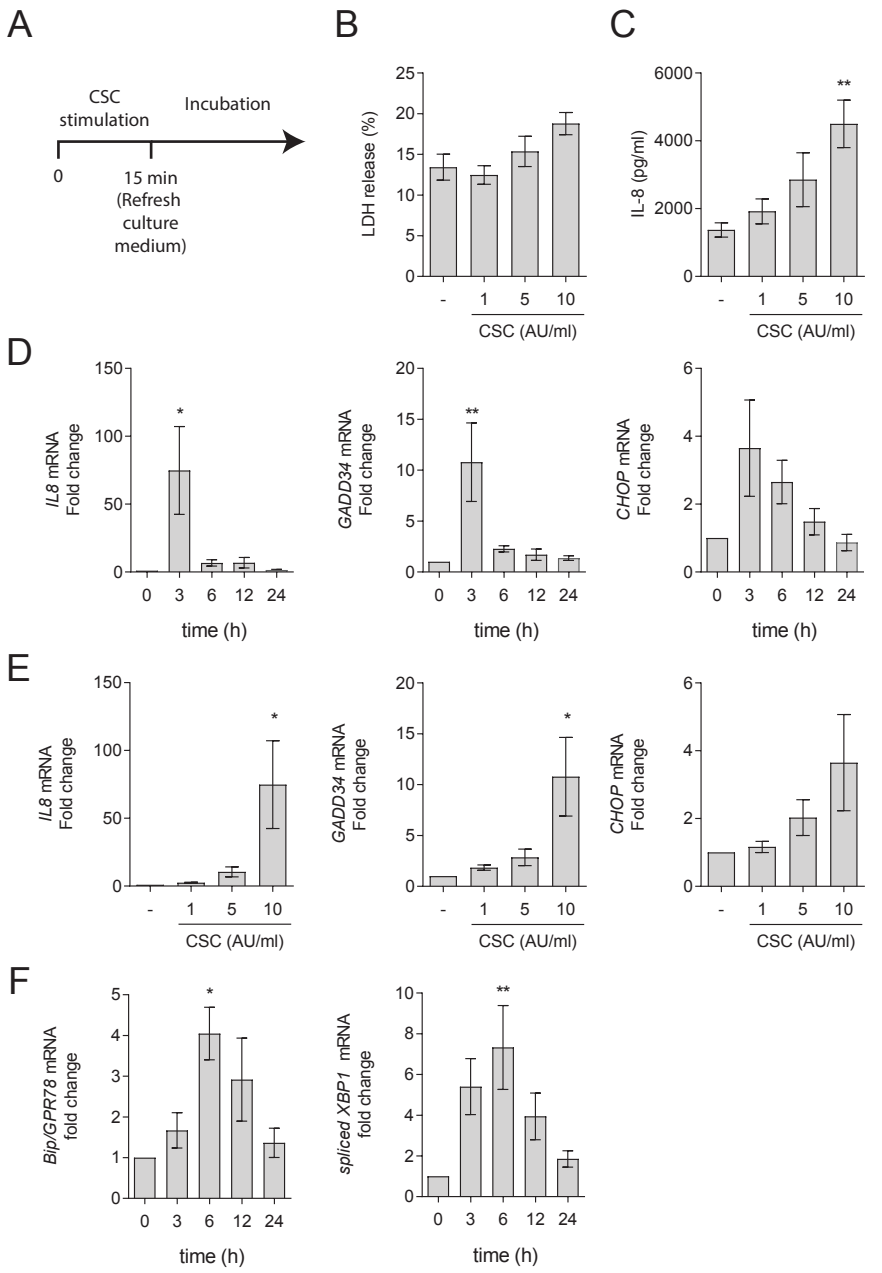

Figure 4. Effect of CSC on ISR activation in undifferentiated S-PBEC. (A) Schematic of experiments with CSC pulse exposure of S-PBEC cultures. (B) Assessment of the cytotoxic effect of 1-10 AU/ml CSC on S-PBEC after 24 h incubation. Percentage LDH release was used as measurement of cytotoxicity. (C) Assessment of IL-8 protein release in the cell culture supernatant of S-PBEC stimulated with 1, 5 and, 10 AU/ml CSC and incubated for 24 h. (D) Assessment of *IL8*, *GADD34* and *CHOP* mRNA expression at different time points (3-24 h) after 10 AU/ml CSC exposure of S-PBEC. mRNA expression is shown as fold chance compared to control-treated cells. (E) Assessment of

IL8, GADD34 and *CHOP* mRNA expression in S-PBEC after stimulation with 1, 5, and 10 AU/ml CSC exposure and 3 h incubation. mRNA expression is shown as fold chance compared to control-treated cells. (F) mRNA expression of BiP/*GRP78* and *sXBP1* at different time points (3-24 h) after 10 AU/ml CSC exposure of S-PBEC. mRNA expression is shown as fold chance compared to controls. Results are shown as mean ± SD using cells of at least 3 independent donors. Analysis of differences was conducted using a one-way ANOVA with a Bonferroni *post-hoc* test . * $p < 0.05$ and ** $p < 0.01$.

be mediated by oxidative stress. eIF2α^{AA} and eIF2α^{SS} MEFs were treated with the anti-oxidant N-acetylcysteine (NAC) and CSC in combination. CSC-induced expression of GADD34 was inhibited by NAC in both eIF2α^{AA} and eIF2α^{SS} MEFs (Figure 5C). In contrast, the ER stress activator thapsigargin induced GADD34 expression only in ISR-competent eIF2α^{SS} and this induction was insensitive to NAC (Figure 5C). This finding suggests that CSC can modulate the ISR via oxidative stress-induced GADD34 expression.

DISCUSSION

In the present study, we have used two complementary cigarette smoke exposure models to show that that short-term exposures of airway epithelial cells to cigarette smoke results in activation of the ISR. Whole cigarette smoke rapidly caused ISR activation in ALI-PBEC, which declined at later time points. Moreover, during the recovery period we observed that the kinetics of eIF2α dephosphorylation and recovery of protein synthesis corresponded with that of GADD34 protein expression. This suggests that the transient activation of the ISR by smoke is attenuated by GADD34.

We have previously shown that acute exposure to cigarette smoke caused a transient disruption of the epithelial barrier integrity in ALI-PBEC (18). Furthermore, we have shown that cigarette smoke transiently impaired epithelial wound repair by reducing cell migration (21). Interestingly, we found that recovery of epithelial barrier and wound repair functions were occurred 6 hours after exposure, which corresponds with the kinetics of GADD34 protein expression and the recovery of protein translation it mediates. It is therefore tempting to speculate that GADD34-dependent recovery of protein translation might be responsible, at least in part, for the recovery of ALI-PBEC cultures following smoke exposure. However, we currently lack conclusive evidence that proves that GADD34 expression is cytoprotective in this context.

We observed more pronounced expression of *CHOP* and *GADD34* mRNA in ALI-PBEC cultures from COPD patients when compared to non-COPD controls, and a correlation between gene expression and lung function parameters. This finding is in line with our earlier study that detected an ATF4 gene signature within the airway of individuals with COPD, although *GADD34* and *CHOP* were not part of the transcriptome data (16). Other genes, such as *PTGS2*, *DUSP5* and *cFOS*, that were found to be increased in COPD airways (16), were not different between COPD and non-COPD cultures exposed to CS in the current study. This is likely reflects the existence of additionally signaling pathways acting upon epithelial cells *in vivo* that do no persist *ex vivo*. More pronounced activation of ISR in COPD cultures may report and innate increased susceptibility to cigarette smoke of some individuals, although

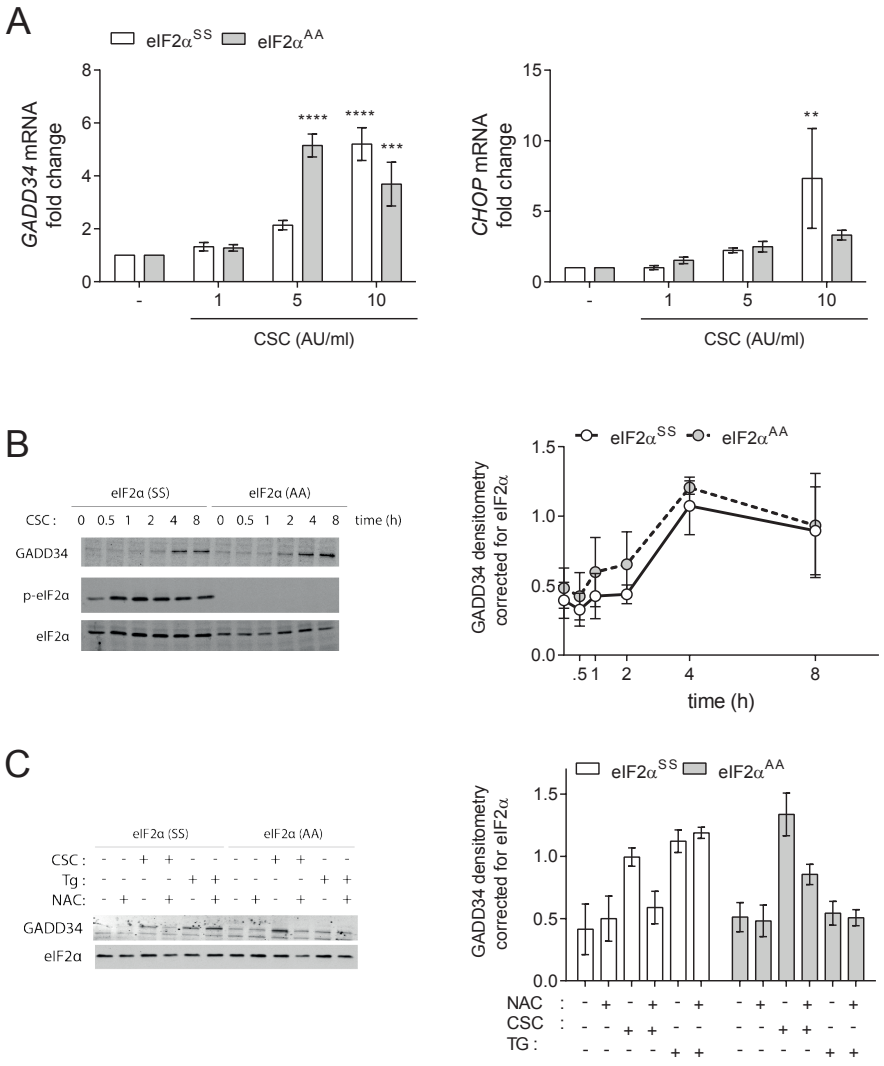

Figure 5. ISR-independent expression of GADD34 in cigarette smoke condensate (CSC) exposure of MEFs. (A) Assessment of GADD34 and CHOP mRNA expression in eIF2α^{AA} and eIF2α^{SS} MEFs stimulated with 1,5 or 10 AU/ml CSC after pulse stimulation and followed by 24 h incubation. Results are shown as fold change in mRNA compared to control-treated cells. (B) Assessment of GADD34 protein expression in eIF2α^{AA} and eIF2α^{SS} MEFs stimulated with 10 AU/ml CSC after pulse stimulation and followed by incubation at different time points. Results were quantified by densitometry using total eIF2α as loading control. (C) Assessment of the effect of N-acetyl cysteine (NAC) on CSC and thapsigargin (Tg)-induced expression of GADD34 in eIF2αAA and eIF2αSS MEFs after 8 h incubation. Results were quantified by densitometry using total eIF2α as loading control. Results are shown as mean ± SD of at least 3 different experiments. Analysis of differences was conducted using a one-way ANOVA with a Bonferroni *post-hoc* test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0005$.

this might be considered out of keeping with a proposed protective role for the ISR during exposure to smoke. However, the chronicity stress has major effects both on the output of the ISR and on the pathological consequences of smoke, which may account for some of these apparent disparities (24). For example, it is plausible that ISR-independent expression of GADD34 caused by oxidative stress may enhance the recovery of protein synthesis that would otherwise by inhibited by CS. Alternatively, a cytoprotective effect of the ISR during acute cigarette smoke exposure might support the persistence of other airway epithelial cells functions that contribute to COPD pathogenesis. It has been shown, for instance, that CHOP can regulate airway epithelial expression of CXCL8/IL-8 in airway epithelial cells (25, 26). Moreover, the recovery of protein translation mediated by GADD34 has been shown to enable for the synthesis of innate immune mediators including IL-6, interferon-α and -β (27, 28). In addition, it has been shown that GADD34 knockout mice display reduced lung inflammation upon exposure to the cigarette smoke component acrolein (29). Overall, these studies suggest that both CHOP and GADD34 may contribute to inflammatory responses, and therefore increased expression in COPD airway epithelial cells may further enhance airway inflammation in response to cigarette smoke.

Similar to airway epithelial cells, CSC also increased expression of CHOP and GADD34 in MEFs. This is in line with the non-cell type specific activation of the ISR by cigarette smoke, which has been shown in other cell types besides airway epithelial cells (8, 30). When examining the role of ISR activation in this response, using cells expressing a non-phosphorylatable mutant of eIF2α, we demonstrated that GADD34 expression occurred independent of the ISR. This alternative pathway is dependent upon oxidative stress and can be inhibited by antioxidants. This effect could be reproduced in the airway epithelial cell line 16HBE (Supplementary Figure 2A), suggesting conservation of this mechanism in airway epithelial cells. Previous reports have shown that activation of the p38 MAPK pathway led to increased expression of GADD34 (31). Indeed, CSC-induced activation of p38 in airway epithelial cells was shown to be oxidative stress-dependent (20), and therefore it is possible that this signaling axis is involved in the ISR-independent expression of GADD34. In experiments using the airway epithelial cell line 16HBE, we observed reduced CS extract-induced GADD34 protein expression upon inhibition of p38 (Supplementary Figure 2B). However, further research is required to demonstrate this in primary airway epithelial cells. As oxidative stress has an important role in the induction of inflammatory responses in airway epithelial cells, it can be further speculated that the oxidative stress-mediated expression of GADD34 may contribute to this response. Moreover, persistent GADD34 expression induced by oxidative stress resulting from prolonged exposure to cigarette smoke may contribute to cell death by completely inhibiting activation of the ISR.

In summary, we have shown that ISR activation is part of the response of airway epithelial cells to cigarette smoke. This response is more pronounced in COPD airway epithelial cells and correlates with disease severity, suggesting a role of the ISR in COPD. Moreover, an oxidative stress-dependent and ISR-independent expression of GADD34 suggests the involvement of alternative mechanisms initiated by cigarette smoke exposure that may affect airway epithelial cell functions.

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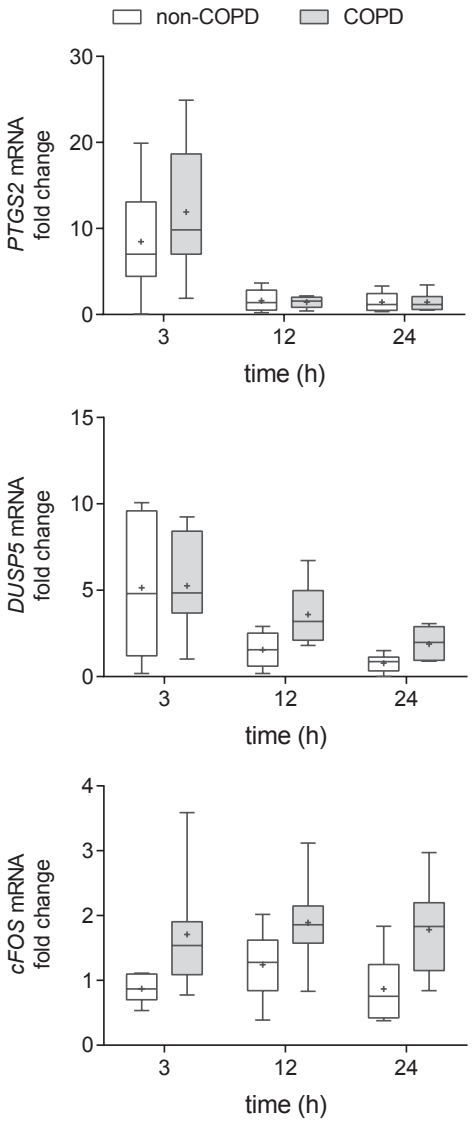
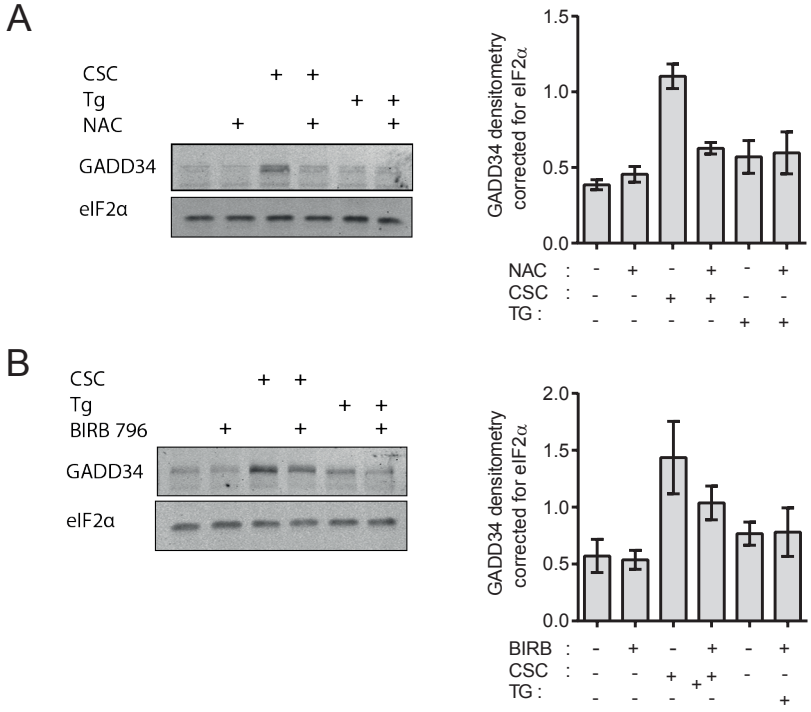
SUPPLEMENTARY FIGURES



Supplementary Figure 1. CS-induced expression of ATF4-related and COPD enhanced target genes in COPD and non-COPD ALI-PBEC. ALI-PBEC from COPD and non-COPD donors were exposed to whole CS and incubated for 3, 12, and 24 h after which *PTGS2*, *DUSP5* and *cFOS* mRNA expression was determined. n=7 COPD and n=6 non-COPD donors were used in the analysis. Analysis of differences was conducted using a one-way ANOVA with a Bonferroni *post-hoc* test.



Supplementary Figure 2. Effect of N-acetyl cysteine and p38 inhibition on cigarette smoke condensate-induced GADD34 expression in 16HBE. (A) Assessment of the effect of N-acetyl cysteine (NAC) on CSC and thapsigargin (Tg)-induced expression of GADD34 in the airway epithelial cell line 16HBE after 8 h incubation. (B) Assessment of the effect of the p38 inhibitor BIRB 796 on CSC and thapsigargin (Tg)-induced expression of GADD34 in the airway epithelial cell line 16HBE after 8 h incubation. Results were quantified by densitometry using total eIF2α as loading control. Results are shown as mean ± SD of 3 independent experiments.