Cigarette smoke inhibits lung fibroblast proliferation by translational mechanisms

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Abstract

Cigarette smoke is a major cause of chronic obstructive pulmonary disease (COPD) and emphysema. Although cigarette smoke represses cellular proliferation the molecular mechanisms underlying this phenomenon is unknown. CCAAT/enhancer binding proteins (C/EBPs) are key regulators of cell cycle progression, differentiation and pro-inflammatory gene expression, are regulated predominantly at the translational level and may be involved in the pathogenesis of COPD.

Aim of the study was to assess the effect of cigarette smoke on proliferation, the expression and translational regulation of C/EBPα and C/EBPβ, in non-diseased, primary human lung fibroblasts.

Fibroblasts were exposed to cigarette smoke conditioned medium (10% and 20%, 24 hours). Proliferation was determined by [^{3}H]-thymidine incorporation. Protein expression levels were determined by immuno-blotting, and translation was monitored by the novel Translation Control Reporter System (TCRS).

Cigarette smoke significantly reduced fibroblast proliferation and significantly up-regulated full-length C/EBPα and C/EBPβ proteins due to a shift in the translation control of *CEBPA* and *CEBPB* mRNAs. This shift involved the re-initiation of mRNA translation via the regulatory upstream open reading frame (uORF), which coincided with increased IL-8 release and decrease of the functional elastin level.

These findings provide a novel mechanism to understand tissue remodeling observed in the lung of COPD patients.

Keywords:

COPD, smoke, human lung fibroblast, mRNA translation control, C/EBP-alpha and –beta, cell proliferation

Abbreviations

C/EBP = CCAAT/enhancer binding protein

CRT = calreticulin

HA = hemagglutinin

LDH = lactate dehydrogenase

LP = long peptide

SCM = smoke conditioned medium

SP = short peptide

TCRS = translation control reporter system

TOP = tract of oligopyrimidines

uORF = upstream open reading frame

Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by chronic inflammation of the lung and airway remodeling leading to a fixed narrowing of the small airways and destruction of the alveolar wall (emphysema). Cigarette smoke is the main risk factor for the development of the disease [1]. COPD is a global health problem that affects more than 10% of the world population over the age of 40 years [2]. The most widely accepted hypothesis to explain the pathology of COPD and emphysema is an imbalance of proteases and their inhibitors which leads to the degradation of elastin and other structural components of the lung tissue [3, 4]. In another model of the disease, the loss of tissue in end-stage COPD (emphysema) may derive from an imbalance of tissue turnover when proliferation is unable to compensate for tissue loss [5].

Lung fibroblasts maintain the integrity of the lung parenchyma as they contribute to the repair of lung injuries through synthesis and secretion of the main components of the extracellular matrix such as proteoglycans and collagens. Importantly, lung fibroblasts also produce elastin, an essential component of the alveolar extracellular matrix that provides the lung tissue with elasticity [6]. An impaired capacity of lung fibroblasts to execute tissue repair has been found in COPD patients [7]. Cigarette smoke inhibits proliferation of normal fibroblasts [8], induces cellular senescence [9] and inhibits alveolar repair [5].

In the context of tissue maintenance and cell proliferation, CCAAT/enhancer binding proteins (C/EBP) could be of interest. C/EBPs have both stimulatory and inhibitory effects on the proliferation of many cell types and appear to be crucial for the regulation of inflammatory responses. C/EBPs are an important family of transcription factors that regulates cell differentiation, cell cycle progression and the expression of many cytokines and chemokines

relevant to COPD and emphysema [10, 11]. In addition, C/EBPβ was shown to inhibit the transcription of elastin [12] and it was elevated in emphysema lungs [13]. In airway smooth muscle (ASM) cells of asthma patients, the expression of C/EBPα was reduced, due to an impaired translation of the mRNA which associated with a faster proliferation rate [14, 15] and may be related to the hyperplasia of ASM cells in the lungs of asthma patients [16].

In COPD a similar mechanism may underlie a disturbed tissue homeostasis, which may explain the associated parenchymal destruction of the disease. Therefore, we analyzed the effects of cigarette smoke on proliferation rates of primary lung fibroblasts in relation to translational mechanisms involved in the isoform expression of C/EBPα and C/EBPβ, as well as on the expression of IL-8 and elastin.

Methods

Cell cultures

Lung tissue specimens were obtained from the Departments of Thoracic Surgery and of Internal Medicine, Pneumology, University Hospital Basel, Switzerland with the approval of the local Ethical Committees and written consent of all patients. Fibroblast cells were established from small sections of lung parenchymal tissue and grown in RPMI 1640. (Lonza, Basel; Switzerland) supplemented with 5% fetal calf serum (FCS, 8 mM L-glutamine, 20 mM HEPES, 1% MEM vitamine mix) (Gibco, Paisley, UK). Fibroblasts were cultured in $25cm^2$ flasks (Falcon, Becton Dickinson, Switzerland) and cells used in the study were all below passage number 6.

Cigarette Smoke Conditioned Medium (SCM)

SCM was prepared by leading cigarette smoke equivalent of 1 cigarette from a commercially available brand (Gauloises Blondes, Altadis, Switzerland) with a 60ml syringe through 25 ml of RPMI medium in a Shott flask containing an influx and aspiration channel. This cigarette smoke concentration was defined as 100%. The generated SCM was sterile-filtered (0.22μm syringe filter) (Sterilizer PES, Macherey-Nagel, Oensigen, Switzerland). The chemical analysis of the SCM was performed by Gas Chromatography/Mass Spectroscopy full scan and the nicotine content was determined for standardization purposes (EMPA, Dübendorf, Switzerland). Prior to addition of SCM the cells were starved for 24 hours and diluted SCM (0-20%) was added in absence of FCS.

Small inhibitory RNA (siRNA) treatment

Transfection with siRNA for C/EBPα, C/EBPβ or negative control (Santa Cruz Biotechnology, USA) was performed according manufacturer protocol. Cells (60% confluence) were plated into 6 well plates and transiently transfected with siRNA (50nM) for 7 hours. siRNA was added at Day 0 (D0) incubated in FCS-free RPMI thereafter the medium was changed to RPMI (+ vitamin mix + 5% FCS). Cells were incubated for > week. Cell counts were performed at D0, D1, D2, D3, D6, and D9.

Immuno-blot analysis

Equal amounts of proteins were loaded on gradient polyacrylamide gels (4-20%) and immuno-blotting was performed as described previously [17]. Following primary antibodies were used: C/EBPα (AVIVA, San Diego, USA); or C/EBPβ (AVIVA); HA.11 (Covance, Berkley, USA); calreticulin (Santa Cruz Biotech, Santa Cruz, USA); hnRNP E2 (Santa Cruz Biotech); elastin (Millipore, France); α-tubulin (Santa Cruz Biotech). Protein bands were quantified by an image analysis system (ImageJ) and protein expression was normalized to α-tubulin [17].

Translation Control Reporter System (TCRS)

Cells were transfected with TCRS (gift from Dr Calkhoven, Jena, Germany) in Tfx^{TM}-50 reagent (Promega, Madison, USA). Cells (70% confluence) were incubated with 2.5 μg/well TCRS construct for 1 hour (37°C). Then medium containing 5% FCS was added and the cells were incubated for 48 hours. Before further experiments, the cells were kept for 24 hours in starving conditions. The ratio of the short protein (SP) to the long protein (LP) was determined by immuno-blot and analyzed by image analysis as previously described [15].

Proliferation assay

Fibroblasts were seeded in a 96 well plates (4,000 cells/well, 60% confluence) and allowed to adhere in growth medium over night before being serum deprived (24 hours) and stimulated with SCM in the presence of 2 μCi/ml [^{3}H]-thymidine (Perkin Elmer, Boston, USA) at 37°C for 24 hours. After being lysed in 0.1 M NaOH, the DNA was collected onto glass fiber filters and cpm was counted in a Packard TOP COUNT NXT[TM] [17].

Membrane integrity assay

The cytotoxic effect of SCM was determined by membrane integrity assay measuring the release of lactate dehydrogenase (LDH) after 24 hours according to the manufacturer's protocol (CytoTox-One[TM], Promega, Madison, USA). Cytotoxicity was calculated as LDH increase relative to the untreated control cells.

IL-8 Enzyme-linked immunosorbent assay (ELISA)

Samples of cell culture medium were collected after incubation (2, 4 and 6 hrs) with SCM (20%) and IL-8 ELISA was performed according to the manufacturer's manual (Anibiotech Orgenium Laboratories, Vantaa, Finland).

Statistics

Proliferation data are presented as mean +/- SD, immuno-blot analyses are shown as mean +/- SD after densitometric image analysis (ImageJ, National Institute of Mental Health, Bethesda, Maryland, USA) of at least three independent experiments in cell lines of different subjects. Paired/unpaired Student's t test was performed and P-values < 0.05 were considered significant.

Results

Transient C/EBPα knock-down increased the fibroblast proliferation rate, whereas CEBPβ knock-down reduced the proliferation rate.

To demonstrate the involvement of C/EBPα and C/EBPβ in proliferation of primary human lung fibroblasts, we performed transient knock-down experiments with C/EBPα siRNA and C/EBPβ siRNA. Knocking down C/EBPα resulted in a significant increased proliferation over an interval of 9 days after transfection relative to control siRNA (100%) (figure 1A). In contrast, knocking down C/EBPβ reduced the proliferation rate (figure 1B).

Cigarette smoke conditioned medium (SCM) decreased the proliferation of primary lung fibroblasts.

To assess the effect of SCM on the proliferation capacity of primary lung fibroblasts, cells were incubated for 24h. As shown in figure 2A, SCM dose-dependently reduced the proliferation and the inhibitory effect was significant at all measured concentrations ($p < 0.05$, n=3). Concentration higher than 20% induced cell detachment. To exclude toxic effects on the fibroblasts, we tested the cells for LDH release during incubation with SCM. As shown in Fig. 2B, SCM did not induce LDH release in fibroblasts at 24 hours, demonstrating that SCM does not have significant cytotoxic effects in the concentrations we used throughout our experiments   (10% and 20%, n=4)

SCM increased both C/EBPα and C/EBPβ expression in primary lung fibroblasts.

Primary lung fibroblasts were incubated in absence and presence of two concentration of SCM (10% and 20%). After 24 hours C/EBPα and C/EBPβ were analyzed by immuno-blot. As shown

in figure 3A C/EBPα is expressed as the two full-length isoforms (p40 and p42) in unstimulated cells and increased upon stimulation with SCM reaching significance at the highest concentration (10%, p= 0.9; 20% SCM p< 0.05, n=6). Similar results were found for C/EBPβ (see figure 3B). Here the highest concentration of smoke SCM significantly increased both isoforms of C/EBPβ (10%, p= 0.09; 20% SCM p< 0.05, n=6).

SCM induced translation control does not involve CRT or hnRNP E2.

Figure 4A schematically shows the translation control proteins (hnRNP E2 and calreticulin) involved in translation of CEBPA and CEBPB mRNA. Therefore, we determined calreticulin (CRT) levels in response to SCM. CRT is a repressor protein that reduces CEBP mRNA expression by binding to an internal GC-rich region in the mRNA stem loop [18] (figure 4A). As shown in figure 4B, SCM had no effect on the expression level of the CRT. Therefore, we analyzed the expression of hnRNP E2, another repressor *CEBPA* mRNA translation which binds to the 5' untranslated region [19]. As shown in figure 4C, SCM had no effect on the expression level of the hnRNP E2.

SCM enhances the translation re-initiation of C/EBPA and C/EBPB mRNAs from the first start codon.

Previously we demonstrated that *CEBPA* gene expression is predominantly regulated at the translational level [15]. Here we used the same translation control reporter system (TCRS) to detect changes with respect to translational regulation. The principle of the TCRS is depicted in Fig. 5A. The construct generates a long (p23) and a short (p12) peptide with a hemagglutinin epitope (HA) of which the ratio (p12/p23) is a measure of the translation re-initiation efficiency

from the second start codon in the *CEBPA* and *CEBPB* mRNA open reading frame [20, 21]. SCM significantly decreased the SP/LP ratio ($p < 0.05$; figure 5B), indicating that the re-initiation of translation of *CEBP* mRNAs was shifted to the full-length form of the protein. This coincided with a shift of the C/EBPβ expression from the truncated to the full-length isoform in the primary fibroblasts cell shown in figure 5C, and confirms that SCM controlled the translation of *CEBP* mRNAs via the uORF mechanism.

SCM induced IL-8 and reduced functional elastin levels.

Finally we studied the effect of two important inflammatory and remodeling read-outs, IL-8 and elastin, which are known to be regulated by C/EBPs [12, 13, 26]. As shown in figure 6, SCM significantly increased IL-8 expression ($p < 0.05$, n=3; figure 6A), and reduced the ratio between functional elastin levels and elastin break-down products from 0.40 to 0.22 (n=3, figure 6B).

Discussion

In this study, we showed for the first time that SCM has a modulatory effect on the translation control mechanisms of the cell cycle regulators and transcription factors C/EBPα and C/EBPβ. This effect is controlled at the translational level and involves the regulatory upstream open reading frame (uORF) of *CEBPA* and *CEBPB* mRNA, leading to an increased full-length protein expression and therefore suppresses proliferation. In contrast, SCM has no effect on the expression level of CRT and hnRNP E2, two important proteins involved in *CEBP* mRNA translation regulation. We hypothesize that in cigarette smoke susceptible subjects this smoke-induced translational modulation of *CEBP* mRNAs may contribute to an impaired lung tissue renewal process, a diminished protein turn-over, and may underlie the inflammation and destruction of the lung parenchyma.

A cigarette smoke-induced modulation of pro- and anti-proliferative signals may hence provide a novel mechanism to explain many features of COPD and emphysema. C/EBPs are important regulator of cell cycle progression and proliferation and a disturbed balance in their expression has been linked to many proliferative disorders [10, 11]. C/EBPα and -β are modular proteins containing a C-terminal basic leucine-zipper domain required for homo- and hetero-dimerization and binding of the DNA in CCAAT-type-cis-regulatory motifs in responsive genes and a N-terminal sequences containing trans-activation domains [11]. Different full-length or truncated isoforms stem from one single mRNA transcript due to alternative translation initiation at different translation start sites within the open reading frame of the *CEBPA* and *CEBPB* mRNAs. The translation initiation of both C/EBPs is predominantly regulated by a small upstream open reading frame (uORF) [21]. Importantly, the truncated C/EBP isoforms miss the trans-activation

domain and are therefore unable to activate gene transcription and inhibit cell proliferation. In airway smooth muscle cells of asthma patients an impaired translation of the *CEBPA* mRNA was observed, which coincided with lower levels of CEBPα protein and increased levels of IL-6 [15, 22]. In a similar way, smoke exposure may decrease the proliferation due to a translational shift of *CEBP* mRNA expression levels to the full-length protein isoforms.

Here, we focused on three well-known mechanisms [18, 19, 21], which control the translation of *CEBPA* and *CEBPB* mRNA. First, we tested two proteins that repress *CEBP* mRNA translation. Calreticulin (CRT) inhibits the translation of the *CEBPA* and *CEBPB* mRNAs into protein due to a direct interaction of CRT to an internal stem loop of both *CEBPA* and *CEBPB* mRNA [18]. Alternatively, the protein hnRNP 2E can bind to the 5' untranslated region of the *CEBPA* mRNA and inhibit its translation, which has been shown in the context of leukemia [19]. Here we did not observe an effect of SCM on the expression level of both CRT and hnRNP 2E, indicating that a distinct mechanism is involved.

To further elucidate the mechanism underlying the expression of C/EBPα and C/EBPβ proteins by cigarette smoke exposed fibroblasts, we used a novel translation control reporter system (TCRS), which senses translation control and is regulated via a uORF present in both *CEBPA* and *CEBPB* mRNA [20, 21]. The TCRS generates two peptides (p12 and p23), and their expression ratio (p12/p23) is a quantitative measure of translation initiation efficacy regulated by the uORF [20]. High initiation efficiency at the second start codon results in an increased expression of the small peptide, whereas poor translation re-initiation shifts the expression toward the long peptide (figure 4A). Similarly, low translation re-initiation at the second start codon shifts the expression

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of both C/EBPα and C/EBPβ toward the full-length isoforms. We found that cigarette smoke significantly decreased the p12/p23 (SP/LP) ratio in lung fibroblasts, indicating a shift of translation initiation from the second to the first start codon of the *CEBP* RNAs, which results in the expression of full-lengths proteins. Indeed, we observed a shift from the truncated to the full-length C/EBPβ protein after SCM exposure (figure 5C), which associated with decreased proliferation.

In this report we demonstrated that C/EBPα and -β have opposite effects in fibro-proliferation. The knock-down experiments showed an anti-proliferative role of C/EBPα whereas C/EBPβ is pro-proliferative. C/EBPα plays a decisive role in cell growth arrest, showing an expression pattern that is inversely related to the proliferative state of many cell types [10]. Here we observed that cigarette smoke induced an up-regulation of the full-length C/EBPα, which may account for the strong anti-proliferative effect of SCM on lung fibroblasts. This effect may also underlie the impaired tissue repair and renewal and, eventually, lead to a tissue loss as observed in the emphysema lungs. It should be noted, however, that 60% reduction is already observed at 10% SCM, demonstrating the complex nature of cigarette smoke and indicating the involvement of additional signaling pathways.

The increased C/EBPβ expression after exposure to SCM is in accordance with a mouse model where increased C/EBPβ levels occurred in emphysema lungs following daily exposure to cigarette smoke over a period of 6 months [13]. Interestingly, C/EBPβ was shown to be a negative regulator of elastin expression, a structural component of the lung alveoli [12]. In accordance with this finding, we observed a reduced level of functional elastin (p70) and an

increased level of elastin break-down products in fibroblasts after SCM exposure compared to untreated cells (Figure 6B). As demonstrated, proteolytic degradation of elastin by metallo matrix proteinases (MMPs) resulted in airspace enlargement and development of emphysema [23, 24]. Similarly, our observation that SCM induced the up-regulation of C/EBPβ may explain the impaired re-synthesis of elastin in COPD lung tissue and smoke exposed mice [13]. Together, these two mechanisms may aggravate the parenchymal tissue loss observed in COPD.

It has been shown that the neutrophile chemokine IL-8 is an important inflammatory mediator in COPD [25]. Interestingly, the promoter of the IL-8 gene contains a CCAAT consensus sequence [26]. It can be speculated that the up-regulation of C/EBPβ expression may explain the increased IL-8 release typically found in COPD. In accordance with these findings, we found increased IL-8 levels after SCM exposure (Figure 6A). IL-8 is a strong chemotactic agent for neutrophils, which are the main cells infiltrating the lung during the inflammatory process observed in COPD [1]. In this way, cigarette smoke-induced full-length C/EBP expression may activate pro-inflammatory genes to create an inflammatory environment required to recruit more pro-inflammatory cells in a positive feedback loop.

In summary, our data indicate that exposure of primary lung fibroblasts to cigarette smoke regulates the translation of both *CEBPA* and *CEBPB* mRNA via the regulatory uORF, which then shifts the protein expression towards the full-length C/EBP isoforms. The consequence may be impaired cell proliferation associated with inflammation, which then leads to a reduced regeneration of lung tissue and remodeling of the airways (schematically presented in figure 7).

Insight in aberrant translational control mechanisms of C/EBP expression may potentially lead to new therapeutic strategies for COPD and emphysema.

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Legends

Figure 1. *Proliferation rates in transient knock-downs of C/EBPα and C/EBPβ.* **A.** Proliferation rates of primary human lung fibroblasts transfected with siRNA for C/EBPα (si-α), C/EBPβ (si-β). * p < 0.05 compared to day 0 and day 1 (n=4); ** p < 0.05 compared to day 0 and day 1 (n=4) **B.** Immuno-blot of C/EBPα (top) and β (bottom) in presence of control siRNA, C/EBPα siRNA and C/EBPβ siRNA (as indicated). Bar diagrams represents relative levels of C/EBPα and β relative to controls. (n=3, p< 0.05)
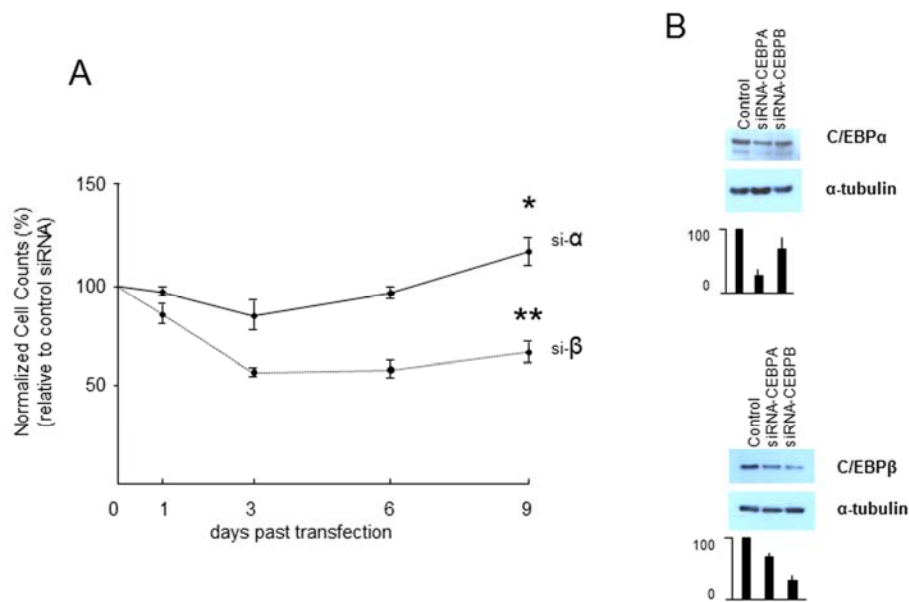
Figure 1 A

Figure 2:

Proliferation characteristics. **A.** SCM significantly inhibited cell proliferation after 24 hours in lung fibroblasts (n=3), *P < 0.05**. B.** LDH release after 24 hours incubation with SCM (n=4). Max. release: LDH value of lysed cells. The results represent the mean ± S.D.
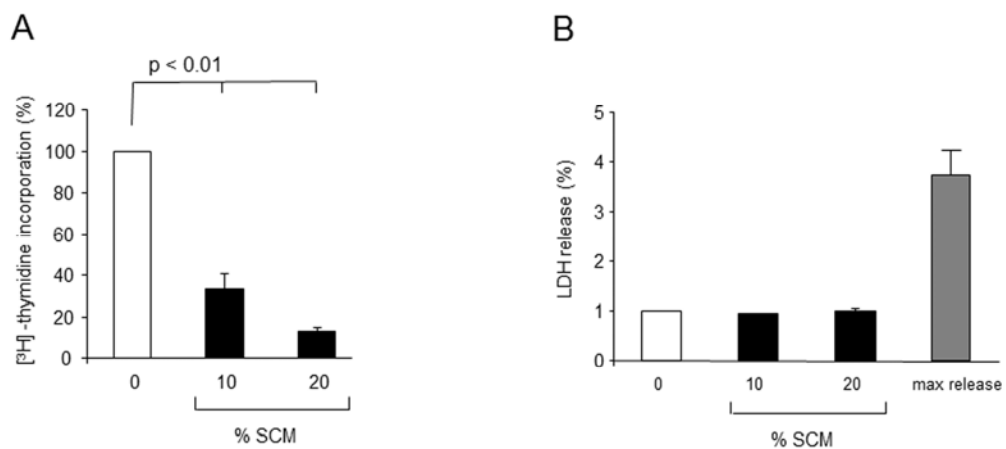

Figure 2

Figure 3:

Immuno-blot analysis of C/EBPα and C/EBPβ. **A.** Representative immuno-blots (duplicates) of the C/EBPα expression in lung fibroblast cells incubated 24 hours in SCM. Bar diagram = densitometry (n=6); * P < 0.05. **B.** Representative immuno-blots (duplicates) of the C/EBPβ

expression in a lung fibroblast cells incubated 24 hours in SCM. Bar diagram = densitometry (n=6); * P < 0.05. The results represent the mean ± S.D.
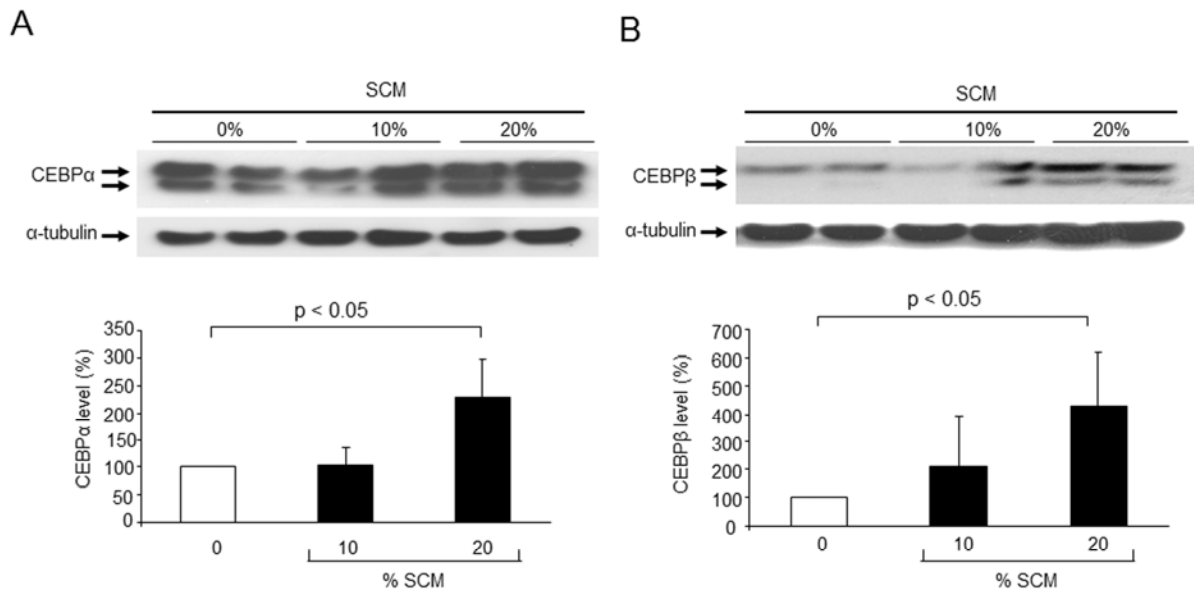

Figure 3

Figure 4:

Immuno-blot analysis of calreticulin (CRT) and hnRNP 2E. **A.** Scheme showing two crucial regulatory proteins involved in the C/EBPα translation control (hnRNP 2E and calreticulin)**. B.** Expression of CRT in lung fibroblast cells after exposure to SCM (24 hours, 20%). Bar diagram = densitometry (n=3). **C.** Expression of hnRNP 2E in lung fibroblast cells after exposure to SCM (24 hours, 20%). Bar diagram = densitometry (n=3). * P < 0.05.
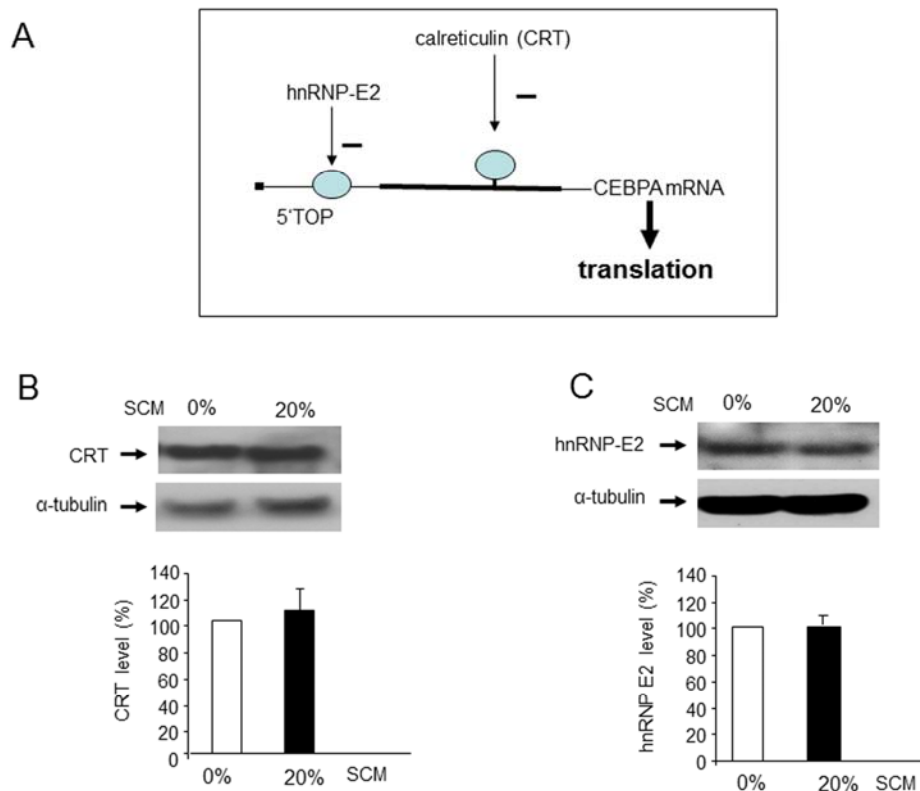
The results represent the mean ± S.D.

Figure 4

Figure 5:

Immuno-blot analysis of translation control of C/EBPα and C/EBPβ. **A.** Schematic representation of the translation control reporter system (TCRS). Impaired translation leads to lower re-initiation at the second start codon and as a consequence to decreased expression of the short peptide (uORF: regulatory upstream open reading frame). **B.** Representative immuno-blot of the expression of the short (p12) and long peptide (p23) in primary lung fibroblasts exposed for 24 hours to SCM (20%). The figure shows duplicates of two independent experiments. Calculated p12/p23 ratios of three different fibroblast cell lines. All experiments were performed in duplicates. The results represent the mean ± S.D. **C.** The shift from the truncated to the full-

lengths C/EBPβ after exposure to SCM (20%) confirms the translation control mechanism as described by Calkhoven et al. [21].
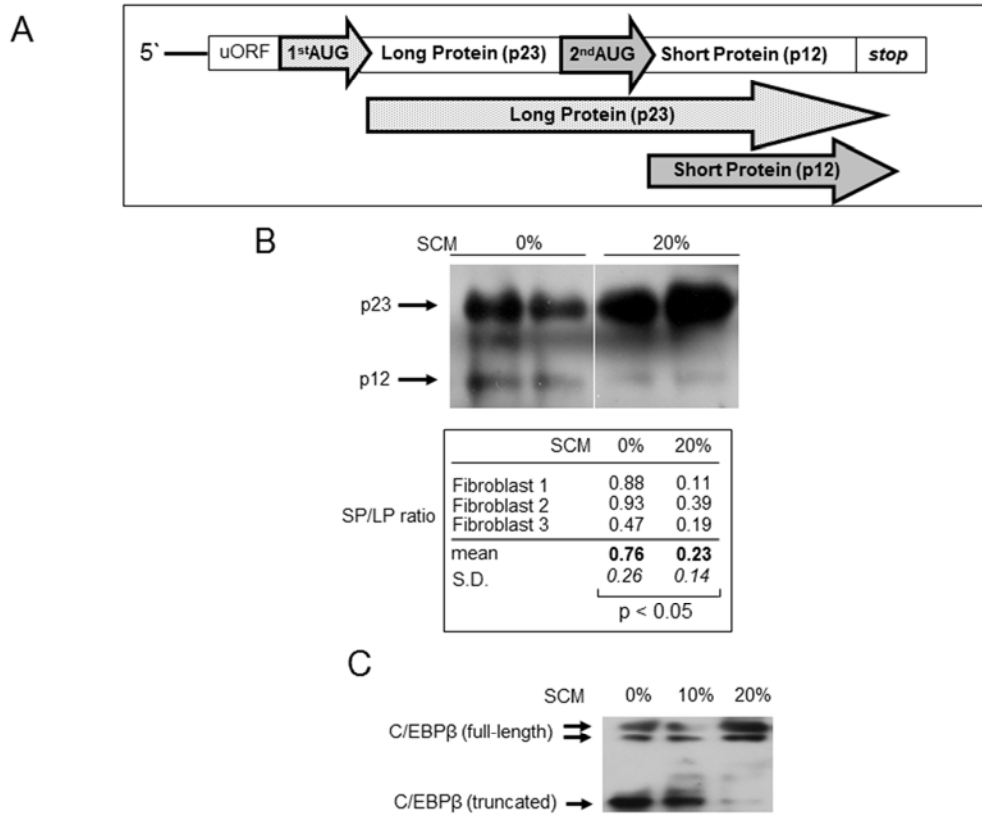

SCM	0%	20%
Fibroblast 1	0.88	0.11
Fibroblast 2	0.93	0.39
Fibroblast 3	0.47	0.19
mean	**0.76**	**0.23**
S.D.	*0.26*	*0.14*

Figure 5

Figure 6:

Pro-inflammatory characteristics and immuno-blot analysis of elastin of primary lung fibroblasts exposed to smoke conditioned medium (SCM)

A. SCM (20%) significantly increased IL-8 secretion over a time interval of 6 hours in primary lung fibroblasts; values normalized to time point 0 (=100%), (n=3). **B.** Representative immuno-blot (n=3) of functional and degraded elastin expression in lung fibroblast cells incubated for 24 hours in SCM (20%).
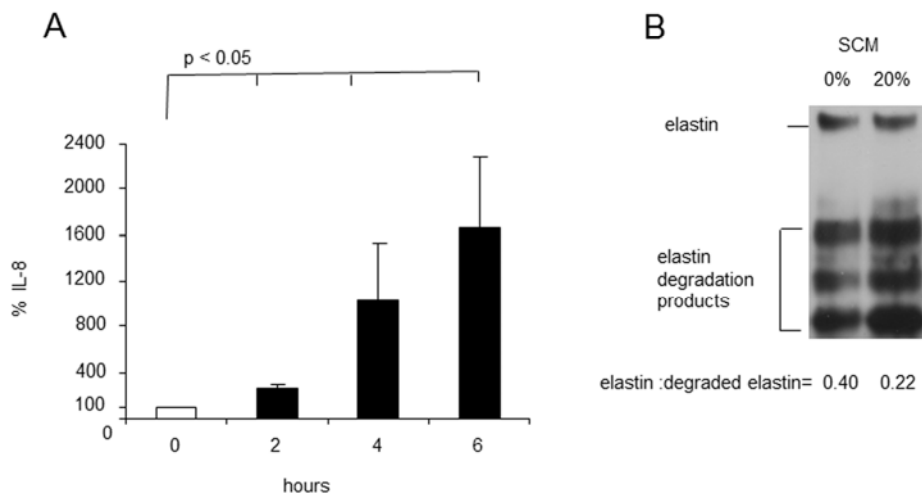
Figure 7:

Schematic overview of putative mechanisms involved in exposure to cigarette smoke and how this might be relate to the pathogenesis of COPD/emphysema.
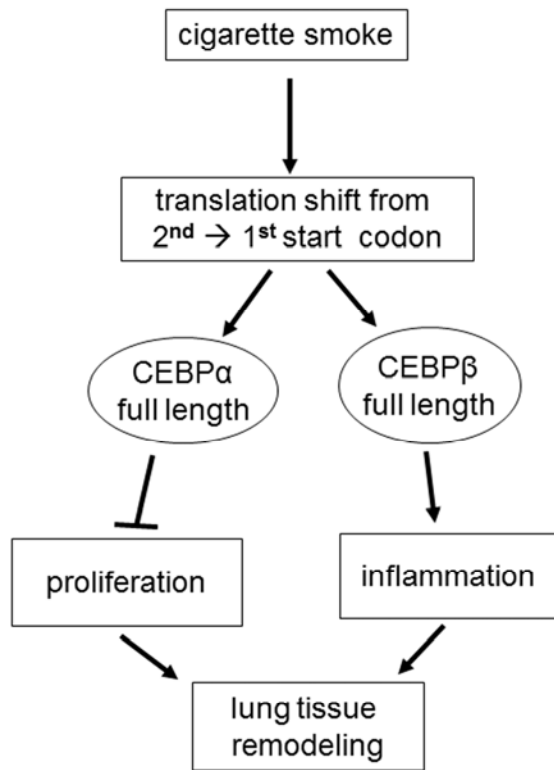

Figure 7