

Impaired translation of CCAAT/enhancer binding protein α mRNA in bronchial smooth muscle cells of asthmatic patients

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Background: Bronchial smooth muscle (BSM) cells of asthmatic patients have an impaired expression of CCAAT/enhancer binding protein (C/EBP) α , which is associated with increased proliferation.

Objective: We sought to assess the translational regulation of *CEBPA* mRNA in cultured BSM cells of healthy control subjects (n = 11) and asthmatic patients (n = 12).

Methods: Translation efficiency was studied by using a translation control reporter system driven by the control elements present in the *CEBPA* mRNA. Translation efficiency was determined by the ratio of 2 artificial hemagglutinin (HA.11) proteins: p23 and p12. We also analyzed levels of proteins that control translation of *CEBPA* mRNA, namely heterogeneous nuclear ribonucleoprotein E2, calreticulin, eukaryotic translation initiation factor (eIF4E), and 4E binding protein.

Results: Compared with healthy control subjects, BSM cells of asthmatic patients proliferate faster (2.1-fold) and are primed for IL-6 secretion. Real-time RT-PCR showed that BSM cells of asthmatic patients express normal levels of *CEBPA* mRNA, whereas they express lower levels of C/EBP α (p42). Transient transfections with the translation control reporter system construct showed a disturbed p12/p23 ratio in BSM cells of asthmatic patients relative to healthy control subjects, which coincided with lower levels of eIF4E.

Conclusion: BSM cells of asthmatic patients have normal levels of *CEBPA* mRNA but inadequately reinitiate the translation into C/EBP α . Impaired translation control upstream of eIF4E might underlie the observed increased proliferation and

priming of BSM cells of asthmatic patients. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Asthma, bronchial smooth muscle cell hyperplasia, C/EBP α , messenger RNA translation

Pulmonary inflammation and airway wall remodeling are characteristic pathologic features of asthma and are assumed to result from chronic airway inflammation.¹ Therefore asthma research has mainly focused on the inflammatory component of the disease. In particular, the studies on mast cells, eosinophils, and T lymphocytes have contributed to our knowledge on airway inflammation and how it aggravates asthma symptoms. The remodeled airways of asthmatic patients display an increase in bronchial smooth muscle (BSM) mass for which the cause is unknown but with a major effector function in airway constriction.² Recent studies showed that bronchial thermoplasty (a novel bronchoscopic strategy to reduce the mass of BSM) markedly improved asthma control in subjects with mild or severe asthma.³ These findings are in line with our proposition that infiltrating cells of the immunologic compartment might not be the cause of asthma but rather that asthma might be regarded as the result of aberrant behavior of the BSM cell because of an imbalanced expression of alternative forms of CCAAT/enhancer binding protein (C/EBP) α , a transcription factor that controls cell proliferation, differentiation, and inflammation. The biologic effects of C/EBP α are exerted by a full-length (p42) isoform and counteracted by a truncated isoform (p30) that is generated at the translational level.⁴ We and others reported that BSM cells obtained from asthmatic patients proliferate faster than their nonasthmatic counterparts,^{5,6} have an activated phenotype characterized by more severe contraction,^{2,7} and demonstrate increased mediator production.⁸ The faster proliferation rate observed for BSM cells of asthmatic patients was associated with diminished expression of C/EBP α .^{8,9} Understanding the origin of the hyperreactive (primed) phenotype of the asthmatic BSM cell might prove valuable for developing novel therapeutic strategies. Therefore we investigated the translational regulation of *CEBPA* mRNA and the proliferative response of primary human nontransformed BSM cell lines obtained from asthmatic patients and nonasthmatic control subjects.

METHODS

Tissue specimens and cell cultures

Lung tissue specimens were obtained from the Department of Pathology, Royal Prince Alfred Hospital; the Cardiopulmonary Transplant Unit and the

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Abbreviations used

BSM:	Bronchial smooth muscle
C/EBP:	CCAAT/enhancer binding protein
4EBP:	4E binding protein
eIF4E:	Eukaryotic translation initiation factor 4E
HA:	Hemagglutinin
hnRNPE2:	Heterogeneous nuclear ribonucleoprotein E2
LP:	Long peptide
mTOR:	Mammalian target of rapamycin
PDGF:	Platelet-derived growth factor
SP:	Short peptide
TCRS:	Translation control reporter system
uORF:	Upstream open reading frame

Department of Pathology, St Vincent's Hospital (Sydney, Australia); and the Department of Pathology, University Hospital Basel (Switzerland), after obtaining approval of the local ethics committees and written consent from all patients. BSM cells from control subjects and asthmatic patients were established as previously described.^{5,10} Cells were grown in RPMI 1640 with and without 5% FCS.

Western blot analysis

Equal amounts of proteins were loaded on gradient polyacrylamide gels (4% to 20%), and immunoblotting and DNA binding were performed as described previously.^{5,10} C/EBP α antibodies were obtained from AVIVA Systems Biology (San Diego, Calif), and hemagglutinin (HA.11) antibodies were from Covance (Berkeley, Calif).

RNA extraction and reverse transcription

Cells were starved for 24 hours and then incubated for 0, 6, and 24 hours in RPMI in the absence and presence of 5% FCS or platelet-derived growth factor (PDGF) BB (10 ng/mL). RNA extraction was performed with the RNeasy Mini kit (Qiagen, Hilden, Germany), including the DNA digestion step. The RNA was quantified with a NanoDrop spectrophotometer and reverse transcribed with the Omniscript RT kit (Qiagen).

Real-time PCR

C/EBP α and 18S expression was analyzed with a human C/EBP α Gene Expression Kit (Applied Biosystems, Foster City, Calif) and an 18S Genomic control kit FAM-TAMRA (Eurogentec, Seraing, Belgium), respectively, according to the manufacturers' protocols. Samples were run in duplicates by using a 7500 Fast Real-Time PCR System (Applied Biosystems). Relative C/EBP α levels were expressed as means $\pm$ SEM after normalization to 18S RNA.

Translation control reporter system

We used the translation control reporter system (TCRS) described in detail elsewhere.^{9,10} In brief, to detect translational abnormalities, we used a recently developed TCRS that uses the cis-regulatory upstream open reading frame (uORF) from the *CEBPA* locus to monitor the translation of a dual-reporter gene into 2 unique reporter peptides.^{9,10} The reporter peptides contain a pre-pro-trypsin signal for secretion and 3 distinct immunogenic epitopes for detection and quantification purposes, including a c-myc tag, a FLAG tag, and an influenza HA epitope. Secreted p23 and p12 reporter peptides can be detected with FLAG and c-myc antibodies, respectively. The HA epitope is present on both reporter peptides, and an improved second-generation HA mAb (HA.11) is sufficient to detect both p23 and p12 reporter peptides in a single immunoblot.^{9,10} To test the TCRS, we transiently transfected BSM cells with (1) the TCRS containing the wild-type cis-regulatory uORF from the *CEBPA* locus (TCRS^{WT}); (2) a control TCRS construct with a nonfunctional

uORF (TCRS ^{Δ uORF}), which therefore can only express reporter peptide p23; (3) a TCRS encoding solely the long peptide (LP) p23 (TCRS^{LP}); and (4) a TCRS encoding solely the short peptide (SP) p12 (TCRS^{SP}). After 24 hours, total protein extracts were analyzed for p23 and p12 expression by using a HA.11 antibody. To test the hypothesis that a translational defect underlies aberrant expression of the *CEBPA* gene, we transfected BSM from normal and asthmatic patients with the TCRS^{WT}, which uses the uORF from C/EBP α in a cassette with 2 translation start (ribosome binding) sites. Translation from the first site acts as a control and produces an LP with a FLAG epitope; translation from the second site results in an SP with a Myc epitope. In addition, both LP (p23) and SP (p12) contain an HA epitope and can be detected with HA.11 antibodies. Equally successful transfections result in (1) the FLAG or LP epitope of roughly equal intensity and (2) different amounts of the SP or Myc epitopes depending on translational regulation. To identify translation efficiency by means of Western blot analysis, we used the SP/LP ratio and not the FLAG/Myc ratio (which can be determined in supernatants of BSM cells). A small SP/LP ratio (<1.0) indicates attenuation of translation.^{10,11}

Transfections

Transfections with short interfering RNA or TCRC constructs were performed as described elsewhere.^{10,12}

Data analysis and presentation

For the purpose of statistical analysis, the following groups were compared: control subjects in the absence and presence of 5% FCS and asthmatic patients in the absence and presence of 5% FCS. For each group, data were compared by means of ANOVA (single-factor analysis). Protein analyses are representative of at least 3 independent experiments. Signals were normalized to α -tubulin signals (as indicated).

RESULTS**BSM cells of asthmatic patients are primed for IL-6 release, proliferate faster, and express lower levels of C/EBP α**

In accordance with previous reports,^{3,6,13} BSM cells obtained from asthmatic patients exhibited a significantly increased proliferation rate (2.1-fold) relative to control cells ($P < .05$; Fig 1, A). Time-course experiments showed that, in response to PDGF-BB, the BSM cells of asthmatic patients immediately and significantly enhanced the secretion of IL-6 protein, whereas nonasthmatic cell lines reached the same level only after 24 hours or more of stimulation ($P < .05$; Fig 1, B). The accelerated rate of IL-6 secretion observed in BSM cells of asthmatic patients was associated with decreased levels of C/EBP α p42 compared with those seen in nonasthmatic control subjects (Fig 1, C).

BSM cells of asthmatic patients express normal levels of *CEBPA* mRNA

Real-time RT-PCR revealed that BSM cells of asthmatic patients and control subjects expressed equal levels of *CEBPA* mRNA (Fig 2, A). However, the same BSM cells of asthmatic patients expressed significantly less C/EBP α (both p42 and p30), and the p42/p30 ratio was slightly higher in asthmatic patients compared with that seen in nonasthmatic subjects (2.0 ± 0.21 vs 1.65 ± 0.13 ; Fig 2, B). Together, the data presented in Fig 2, A and B, suggest an impaired or deregulated translation of the *CEBPA* mRNA underlying the observed low levels of the C/EBP α in BSM cells of asthmatic patients.

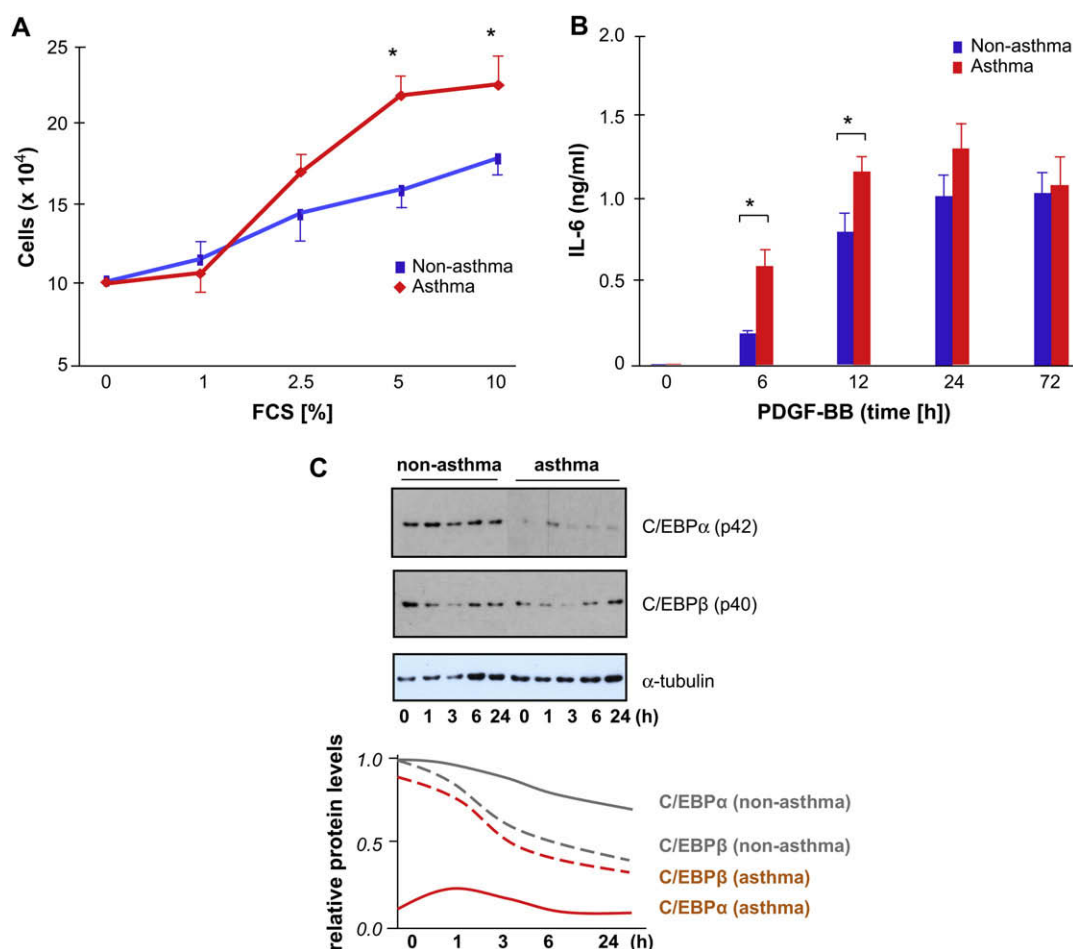


FIG 1. Proliferation and proinflammatory characteristics of BSM cells: asthmatic patients ($n = 11$) and nonasthmatic subjects ($n = 12$). **A**, Proliferation in response to FCS. $*P < .05$. **B**, PDGF-induced IL-6 secretion at 0, 6, 12, 24, and 72 hours. $*P < .05$. **C**, Time course for C/EBP α and C/EBP β protein expression in response to PDGF-BB (10 ng/mL).

BSM cells of asthmatic patients exhibit impaired translation of *CEBPA* mRNA

BSM cells were transfected with the TCRS reporter constructs to detect translational abnormalities. The reporter peptides contain a pre-pro-trypsin signal for secretion and 3 distinct immunogenic epitopes for detection and quantification purposes, including a c-myc tag, a FLAG tag, and an influenza HA epitope (Fig 3, A). In accordance with expectations,^{10,11} p23 (LP) and p12 (SP) bands were observed in BSM cells transfected with TCRS^{WT}, a single LP band was found in cells transfected with TCRS^{ΔuORF} frame or TCRS^{LP}, and a single SP band was found in cells transfected with TCRS^{SP} (Fig 3, B). Next, BSM cell lines obtained from non-asthmatic control subjects and asthmatic patients that express low levels of C/EBP α after FBS treatment were transiently transfected with the TCRS^{WT} and analyzed for C/EBP α (p42/p40), C/EBP α (p30), p23, and p12 after 24 hours. Proliferating BSM cells of asthmatic patients (24 hours, 5% FCS) consistently displayed low levels of the antiproliferative C/EBP α (p42/40) and lacked the p30 truncated variant, which coincided with small SP/LP ratios (Fig 3, C, A1 and A2). Additional transfections with TCRS^{WT} showed that five sixths of BSM cells of asthmatic patients were characterized by small SP/LP ratios, whereas this was only true for one seventh of the cells from control subjects (Fig 3, C-E).

BSM cells of asthmatic patients exhibit lower levels of eukaryotic translation initiation factor

Our data implied a deficiency with respect to the translation of normal levels of *CEBPA* mRNA and prompted us to investigate the signaling pathways involved in translation initiation of *CEBPA* mRNA. As demonstrated in Fig 4, A, BSM cells of asthmatic patients exhibited normal levels of 4E binding protein (4EBP), calreticulin, and hnRNPE2, whereas they demonstrated diminished levels of eukaryotic translation initiation factor (eIF4E; $n = 3$, $P < .05$). A short interfering RNA specific for eIF4E was able to almost completely abrogate the *CEBPA* gene expression in proliferating BSM cells of both asthmatic patients and nonasthmatic subjects (24 hours, 5% FCS; Fig 4, B).

DISCUSSION

In this study we provide further evidence that cultured BSM cells of asthmatic patients have abnormal proliferative characteristics. As reported earlier, BSM cells have low levels of the inhibitory protein C/EBP α (p42), and as a consequence, they might be primed for an accelerated release of proinflammatory cytokines and growth factors that are regulated by C/EBP transcription factors. IL-6 secretion, which is controlled by C/EBP transcription

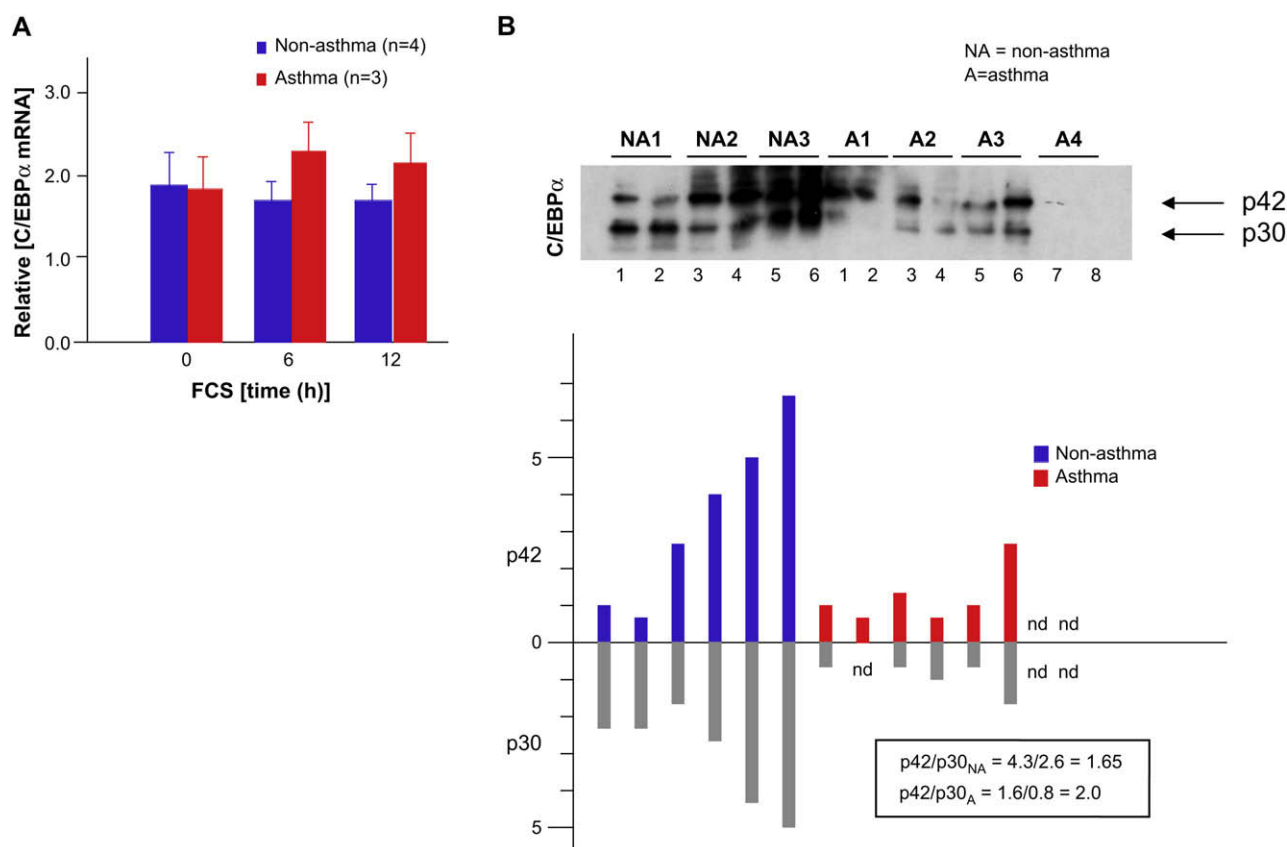


FIG 2. mRNA and protein expression of C/EBPα in BSM cells. **A**, *CEBPA* mRNA expression as determined by means of quantitative real-time PCR after treatment with FCS. **B**, Quantified levels of C/EBPα p42/p30 ratios are expressed in the bar diagram, and those for BSM cells obtained from asthmatic patients and nonasthmatic control subjects are shown in the box insert. nd, Not detectable.

factors,^{14,15} provides an easy readout for the functional effects of inefficient translation of *CEBPA* mRNA. Because all C/EBP isoforms compete with each other in promoter binding, it is the balance between the isoforms that regulates the ultimate effect.⁴ We hypothesized that an altered expression of C/EBPα should be reflected in variable levels of secreted IL-6. IL-6 secretion time-course experiments showed that in response to PDGF-BB, the BSM cells of asthmatic patients immediately and significantly enhanced the secretion of IL-6 protein, whereas nonasthmatic cell lines reached the same level only after 24 or more hours of stimulation. Previously, we showed that impaired C/EBPα levels in asthmatic BSM cells did not affect end-stage IL-6 production levels after 48 hours of stimulation with FCS.¹³ Here we show that BSM cells of asthmatic patients were primed to rapidly secrete IL-6. This observation might be related to a diminished expression of C/EBPα in BSM cells of asthmatic patients. We surmise that in normal resting cells the promoter of the *IL6* gene is occupied by C/EBPα, keeping the gene in an inactive state. After the addition of FCS, C/EBPα is replaced by C/EBPβ, which activates the gene in a similar way as has been described for the activation of the *COX2* gene.¹⁶ If lower levels of C/EBPα are present, C/EBPβ might already occupy the promoter of the *IL6* gene and accelerate transcription.

Earlier, we reported that C/EBPα could not be detected in BSM cells of asthmatic patients¹³ and that about one third of the BSM cell lines of asthmatic patients studied expressed low levels of

distinct isoforms of C/EBPα.⁹ Using a novel C/EBPα antibody (AVIVA), we observed that the detection of C/EBPα in BSM cells of asthmatic patients was dependent on the epitope recognized by the antibody. Antibodies that recognize an epitope of an internal region of C/EBPα (p42) of rat origin (Santa Cruz Biotechnology) were used in our previous studies^{9,13} and did not detect human C/EBPα proteins (p42 and p30) in BSM cells of asthmatic patients. In contrast, antibodies raised against human C/EBPα (AVIVA) readily detected both C/EBPα (p42/40) and C/EBPα (p30) isoforms in protein extracts of BSM cells from both asthmatic and nonasthmatic subjects. In accordance with our previous reports, however, we found significantly lower levels of all C/EBPα isoforms (p42/40 and p30) in BSM cells obtained from asthmatic patients, which was further downregulated after incubation with 5% FCS (Fig 3, C). These findings suggest that the structure or shape of C/EBPα isoforms found in BSM cells of asthmatic patients might be different from that of control cells.

C/EBPα proteins are modular proteins containing a C-terminal basic leucine-zipper domain required for dimerization and DNA binding in CCAAT-type cis-regulatory motifs in responsive genes and N-terminal sequences involved in the transactivation of genes and the regulation of the cell cycle.¹⁷ C/EBPα can be expressed as a full-length protein (p42/p40) with an inhibitory effect on cell proliferation but also as a truncated (p30) polypeptide lacking the inhibitory function.¹⁸⁻²¹ C/EBPα (p42) and C/EBPα (p30) arise from 1 unique *CEBPA* mRNA through different translation

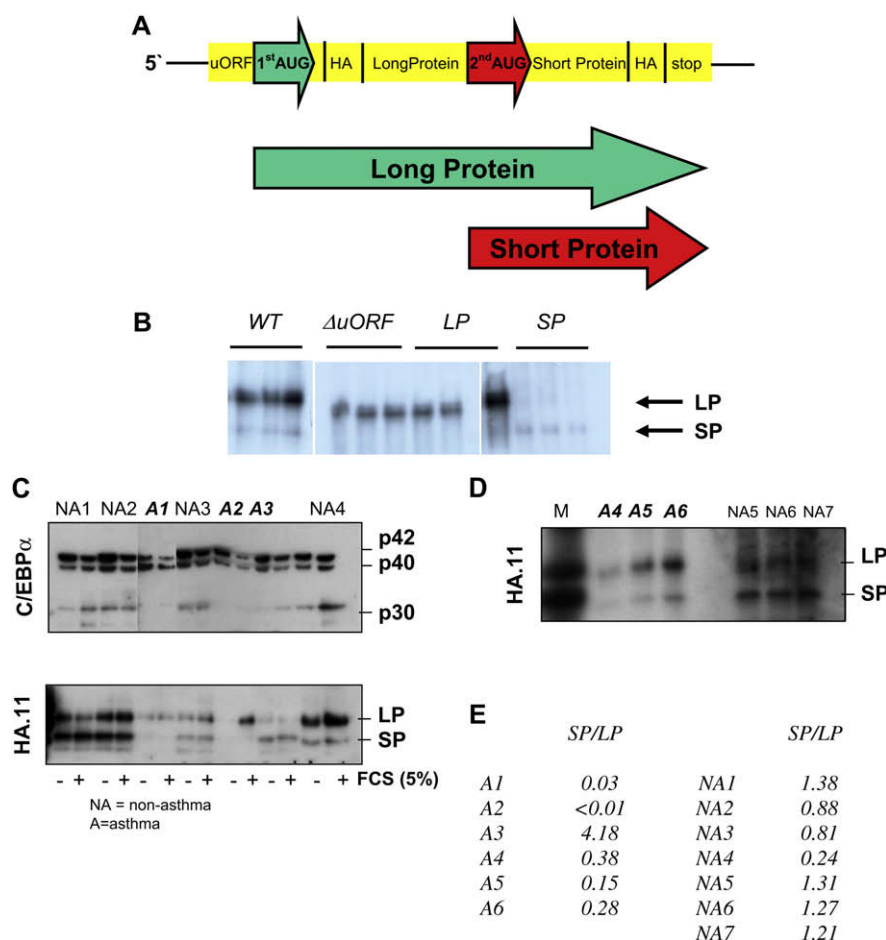


FIG 3. Western analysis of impaired translation of C/EBPα in BSM cells of asthmatic patients. **A**, Schematic representation of the TCRS. For construct details, see the Methods section. **B**, Normal BSM cells transiently transfected with TCRS^{WT}, TCRS^{ΔuORF}, TCRS^{LP}, and TCRS^{SP}. **C**, C/EBPα isoforms and SP/LP ratios expressed in TCRS^{WT}-transfected BSM cells. For comparison, A1 required longer exposure (*insert*). **D**, SP/LP expression in TCRS^{WT}-transfected BSM cells. M, Marker. **E**, The corresponding ratios.

initiation sites. Enhanced expression of truncated C/EBPα isoforms caused abnormal proliferation characteristics in several cell types and the development of inflammatory disorders.²² Full-length C/EBPα (p42/p40) isoforms prevail in differentiating cells and reflect proliferation arrest. Truncated C/EBPα (p30) interfered with terminal differentiation and induced cell transformation^{21–23} but did not have the antimetabolic effect of full-length C/EBPα.²¹ The ratio of full-length and truncated C/EBPα isoforms is strictly regulated by a specific class of cis-regulatory sequences in the *CEBPA* mRNA, known as uORFs. The uORF present in the 5' untranslated region of the *CEBPA* mRNA senses subtle changes in the activity of the translation machinery as a response to intracellular signaling cascades activated by external stimuli, such as fluctuations in nutrients, growth factors, mitogens, and factors that induce differentiation.²² We hypothesize that a deregulation of C/EBPα at this level occurs in BSM cells of asthmatic patients.

Moreover, in BSM cells of nonasthmatic patients, the expression of full-length C/EBPα (p42/p40) and reporter proteins was increased on serum treatment, whereas in BSM cells of asthmatic patients serum treatment rapidly downregulated the expression of both C/EBPα (p42/p40) and reporter proteins. In addition, these

asthmatic patient–derived cells were unable to properly translate C/EBPα (p30). The almost complete absence of p12 (SP) proteins from BSM cell lines of asthmatic patients confirmed an impaired translation in asthmatic patients only (Fig 3, C, lower panel, and Fig 3, D). In many cell types low levels of C/EBPα (p42/p40) relative to C/EBPα (p30) (ie, decreased p42/p30 ratio) were associated with hyperplasia and malignant transformation.²² The *CEBPA* mRNA belongs to the group of 5' tract of pyrimides mRNAs, which is translationally regulated by a 5' terminal oligopyrimidine motif,²⁴ and the initiation of translation is the rate-limiting step for the synthesis of protein.²⁵ Our observations in BSM cells of asthmatic patients could explain their increased proliferation rate in terms of dysfunctional translation control of *CEBPA* mRNA. Upstream signals for translation control of *CEBPA* mRNA can be provided by the hnRNPE2,²⁶ the mammalian target of rapamycin (mTOR)–associated pathway,¹⁰ and calreticulin.²⁷ Wiesenthal et al¹⁰ recently showed that the mTOR blocker rapamycin rapidly attenuated the expression of p12 (SP) from the TCRS^{WT}, and the data were interpreted as a failure to reinitiate translation. Likewise, our data obtained in BSM cells of asthmatic patients suggest a failure to reinitiate the translation of *CEBPA* mRNA, which would explain the attenuation of full-length C/EBPα (p42/p40) and the

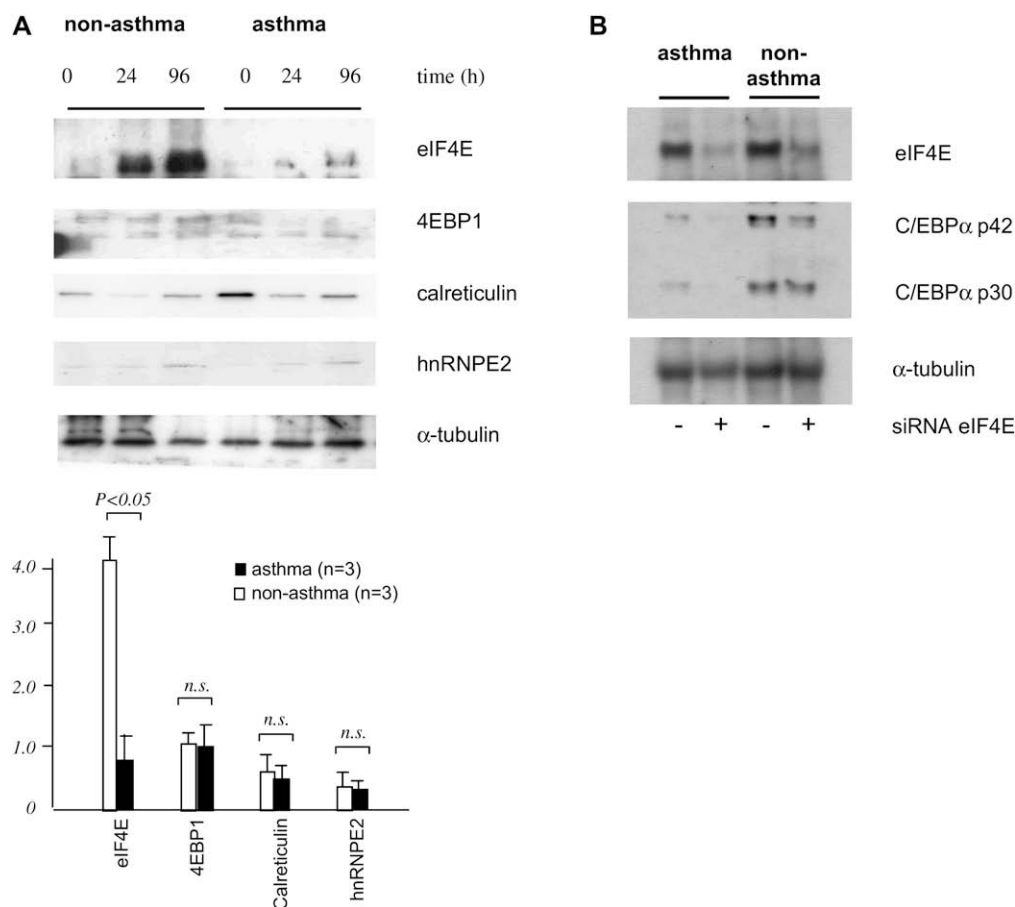


FIG 4. Expression of translation control proteins in BSM cells. **A**, Western blot analysis demonstrates significantly distinct expression patterns of eIF4E/CBPα but not of hnRNPE2, 4EBP1, and calreticulin. The data shown are typical of 3 independent experiments. Quantified protein levels are shown in the bar diagram. *n.s.*, Not significant. **B**, Downregulation of eIF4E and C/EBPα in FCS-stimulated BSM cells by eIF4E short interfering RNA.

complete lack of C/EBPα (p30). Our data therefore suggest an important contribution of a disturbed balance of C/EBPα isoforms to airway remodeling and subsequently to the development, establishment, or both of an asthmatic phenotype. Interestingly, rapamycin completely blocked the proliferation of lung fibroblasts and in a small set of BSM cells.²⁸ In addition, it has been shown that phosphatidylinositol-3 kinase and mTOR activate p70S6K in airway myocytes, leading to the accumulation of contractile apparatus proteins, differentiation, and growth of large, elongated, contractile phenotype airway smooth muscle cells.²⁹

Finally, we found that the attenuated translation of C/EBPα in BSM cells of asthmatic patients was associated with diminished levels of eIF4E, a rate-limiting initiation factor for *CEBPA* mRNA translation that is activated by mTOR through phosphorylation of 4EBP1. In contrast, the expression levels of calreticulin, 4EBP1, and hnRNPE2 proteins that have been reported to be involved in *CEBPA* mRNA translation were not significantly affected. Currently, it is unknown what initiates the disturbed translation control in BSM cells of asthmatic patients, but it might be related to exposure and increased reactivity to allergens. Der p 1, the major allergen present in house dust mite extracts, elicits proasthmatic effects in isolated BSM cells of rat origin,³⁰ and we recently observed that house dust mite allergens reduced C/EBPα protein

levels exclusively in cultured human BSM cells obtained from asthmatic patients but not in BSM cells of nonasthmatic control subjects.³¹ In light of the promising results obtained with bronchoscope-mediated thermoplastic reduction of the mass of BSM cells in the airway walls of asthmatic patients,¹ our findings might open new ways to prevent BSM cells hyperplasia and thus reduce or even cure asthma symptoms through modulation of the appropriate signals leading to balanced expression of full-length and truncated C/EBPα proteins. Considering our findings, less invasive strategies to counteract BSM remodeling should be envisaged.

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Clinical implications: Hyperplasia of BSM cells in the bronchi of asthmatic patients might result from a dysfunctional translation of the *CEBPA* mRNA. Novel strategies to counteract BSM hyperplasia at the translational level should be envisaged.

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