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House dust mite extract downregulates C/EBPα in asthmatic bronchial smooth muscle cells

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ABSTRACT: Reduced translation of *CEBPA* mRNA has been associated with increased proliferation of bronchial smooth muscle (BSM) cells of asthma patients.

Here, we assessed the effect of house dust mite (HDM) extracts on the cell proliferation ([^{3}H]-thymidine incorporation), inflammation (interleukin (IL)-6 release) and upstream translation regulatory proteins of CCAAT/enhancer-binding protein (C/EBP)α in human BSM cells of healthy controls and asthmatic patients.

HDM extract significantly increased IL-6 protein and proliferation of BSM cells of asthma patients only. HDM extract reduced the C/EBPα expression in BSM cells of asthma patients, which coincided with significantly increased levels of calreticulin (CRT) protein, an inhibitor of *CEBPA* mRNA translation. HDM extract elicited both protease-dependent and -independent responses, which were mediated *via* protease-activated receptor (PAR)2 and CRT, respectively.

In conclusion, HDM extract reduced *CEBPA* mRNA translation, specifically in asthmatic BSM cells, and 1) upregulated CRT, 2) activated PAR2, and increased 3) IL-6 expression and 4) the proliferation of asthmatic BSM cells. Hence, HDM exposure contributes to inflammation and remodelling by a nonimmune cell-mediated mechanism *via* a direct interaction with BSM cells. These findings may potentially explain several pathological features of this disease, in particular BSM cell hyperplasia.

KEYWORDS: Asthma, bronchial smooth muscle cells, CCAAT/enhancer-binding protein α, house dust mite extract, mRNA translation

Although bronchial smooth muscle (BSM) hyperplasia is a prominent feature of airway remodelling in asthma and may be linked with the severity of the disease, its mechanism is still of unknown origin. Exposure to allergens during the early years of life leads to a persistent increase in BSM cells by an unknown mechanism, independent of the immune system [1, 2]. Therefore, airway remodelling might not be a secondary event, but rather one of the fundamental pathological causes of asthma [3–5].

In humans, house dust mite (HDM; *Dermatophagoides pteronissinus*) allergens are a major trigger of asthma exacerbation [6, 7]. HDM extract modifies the biology of airway structural cells by its proteolytic activity, thereby disrupting the integrity of the tight junctions between epithelium cells [8]. Once this barrier function of the epithelium is disturbed, as in asthmatic airways, allergens and other particles may easily find their way to deeper areas of the *lamina propria* [9]. HDM-derived compounds, in particular molecules with protease activity, may traverse the epithelium and penetrate even deeper into the airways. Furthermore, the HDM allergen Der p1 directly triggers a change in BSM cell responsiveness and activates the extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase signalling pathway [10]. In this context, it should be noted that HDM extract can elicit protease-dependent and -independent responses [11]. Together, these studies indicate a direct nonimmune-mediated effect of HDM allergens, which induces or contributes to airway wall remodelling and inflammation in asthma.

A persistent abnormality of BSM cells in asthma is a low threshold towards mitogenic stimuli, which is maintained *in vitro* and is associated with decreased levels of CCAT/enhancer-binding protein (C/EBP)α [12, 13]. The lower C/EBPα

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protein levels in BSM cells of asthma patients resulted from impaired translation [14]. C/EBPα is a crucial controller of cell cycle progression, and its protein expression is predominantly regulated by translation through 5′TOP by eukaryotic initiation factor (eIF)4E and heterogeneous nuclear ribonucleoprotein (hnRNP)E2 in humans [14–16]. Translation control might involve a GC-rich sequence in the *CEBPA* mRNA sequence that forms an internal stem loop and is the docking site for calreticulin (CRT), which prevents translation [17]. Only the expression of the full-length C/EBPα maintains normal cell differentiation and function in other cell types [18, 19].

The aim of the present study was to address whether the deficient translation of *CEBPA* mRNA is an intrinsic characteristic of BSM cells in asthma or can be acquired through exposure to external stimuli, such as HDM allergens.

METHODS

Tissue specimens and cell cultures

Lung tissue specimens were obtained from the Dept of Internal Medicine, Pulmonology, University Hospital Basel, Basel, Switzerland with the approval of the local ethical committees and written consent of all patients. BSM cells were established as previously described [12] and grown in RPMI 1640 (Lonza, Basel, Switzerland) supplemented with 5% fetal calf serum (FCS), 8 mM L-glutamine, 20 mM hydroxyethyl piperazine ethane sulfonic acid and 1% modified Eagle's medium vitamin mix (Gibco, Paisley, UK). Neither antibiotics nor antimycotics were added at any time.

Patients

All subjects had atopic asthma, diagnosed as mild to moderate–severe, according to the Global Initiative for Asthma guidelines. Forced expiratory volume in 1 s ranged from 56 to 93% predicted. Mild patients were untreated, whereas moderate–severe patients were undergoing treatment with inhaled steroids, alone or in combination with long-acting β-agonists.

HDM extracts

HDM extract (gift from ALK-Abello, Hørsholm, Denmark) was prepared by dissolving the powder in RPMI 1640 medium at 20 $\mu g \cdot mL^{-1}$, followed by filter sterilisation (0.22 μm) (MN Sterilizer PES; Macherey-Nagel AG, Oensingen, Switzerland). Confluent cells were serum-deprived for 24 h and then stimulated with 10 $\mu g \cdot mL^{-1}$ HDM extract in the presence or absence of 5% FCS over 24 h, in order to allow protein expression. Effects of HDM extracts were determined in presence and absence of FCS, which is mitogenic, thus mimicking both proliferative and inflammatory conditions.

Protein isolation and analysis by immunoblotting

Cellular protein was isolated from confluent cells by dissociation in lysis buffer (62.5 mM Tris–HCl, pH 6.8; 2% sodium dodecylsulfate; 2% β-mercaptoethanol; 10% glycerol), denaturation in sample buffer (3× Laemmli buffer with β-mercaptoethanol) and boiling for 5 min. Equal protein amounts were loaded onto a 4–12% polyacrylamide gel (Pierce Biotech; Thermo Fisher Scientific, Rockford, IL, USA) and size-fractionated by electrophoresis (1 h at 100 V). The gel was sandwiched between two nitrocellulose membranes (Biorad, Reinach, Switzerland) and proteins were transferred (transfer buffer: 0.05 M NaCl; 2 mM Na EDTA; 0.1 mM dithiothreitol; 10 mM Tris–HCl, pH 7.5) overnight (50°C). Protein transfer and equal loading were confirmed by Ponceau staining. The membranes were blocked (10 min) in 3% bovine serum albumin (Roche, Rotkreuz, Switzerland) in 1× PBS with 0.05% Tween-20. The membranes were incubated (1 h) at room temperature with primary antibodies to C/EBPα (AVIVA Systems Biology, San Diego, CA, USA), haemagglutinin epitope (HA.11; Covance, Berkley, CA, USA), CRT (Santa Cruz Biotech, Santa Cruz, CA, USA), smooth muscle cell α-actin (Signet Laboratories, Dedham, MA, USA) or α-tubulin (Santa Cruz Biotech). Membranes were then washed (3×5 min) and incubated (1 h at room temperature) with horseradish peroxidase-labelled, species-specific secondary antibodies (Santa Cruz Biotech). The membranes were washed (3×5 min), incubated (5 min) with ECL substrate (Pierce) and protein bands were visualised on radiographic films (Fuji Film, Luzern, Switzerland). Protein bands were semiquantified using an image analysis system (ImageJ; National Institutes of Health, Bethesda, MD, USA) and protein expression was normalised to α-tubulin as internal control [12].

Translation control reporter system

Cells were transiently transfected with a translation control reporter system (TCRS) using the Tfx™-50 reagent Kit (Promega, Madison, WI, USA). Cells (70% confluence) were incubated with 2.5 μg per well of TCRS construct for 1 h (37°C). Then 5% FCS medium was added and the cells were incubated for 48 h. Before experiments, cells were cultured for 24 h in serum-free medium. The ratio of the short to the long form of the protein was determined by immunoblotting and analysed by image analysis [14].

Small interfering RNA treatment

Transfection with small interfering (si)RNA for CRT or a negative control (Ambion, Austin, TX, USA) was performed according manufacturer's protocol. Cells (60% confluence) were plated into six-well plates and transiently transfected with siRNA (50 nM) for 24 h. Thereafter, fresh RPMI 1640 was added for 24 h and the cells were collected for protein analysis.

Light microscopy

Cells were incubated for 24 h in HDM extract (10 $\mu g \cdot mL^{-1}$) and images were acquired with an Olympus IX50 microscope equipped with Cell^P image software (Olympus Europa GmbH, Hamburg, Germany).

Proliferation assay by [^{3}H]-thymidine incorporation

BSM cells were seeded in 96-well plates (4,000 cells per well, 60% confluence) and allowed to adhere in growth medium overnight before being serum deprived (24 h) and stimulated with HDM extract (1 or 10 $\mu g \cdot mL^{-1}$) in the presence of 2 $\mu Ci \cdot mL^{-1}$ [^{3}H]-thymidine (Perkin Elmer, Boston, MA, USA) at 37°C for 48 h. After being washed with PBS and lysed in 0.1 M NaOH, the DNA was collected onto glass-fibre filters and counts per minute were counted in a Packard TOP COUNT NXT™ (Packard Instrument Company, Meriden, CT, USA) [12].

Cell viability and membrane integrity assay

The cytotoxic effect of HDM extract (10 $\mu g \cdot mL^{-1}$) was determined by a membrane integrity assay, after 24 h in serum-free

medium. The release of lactate dehydrogenase (LDH) was determined according to the manufacturer's protocol (CytoTox-One™; Promega) and fluorescence was assessed at 560/590 nm (Spectramax Gemini XS Microplate Spectrofluorometer; Molecular Devices Corporation, Sunnyvale, CA, USA). Cytotoxicity was calculated as relative LDH increase compared with the untreated control cells.

Interleukin-6 ELISA

Samples of cell culture medium were collected from subconfluent BSM cells after stimulation (24 h) with HDM extract (10 μg·mL^{-1}) and interleukin (IL)-6 ELISA was performed according to the manufacturer's instructions (R&D Systems, Abingdon, UK).

Protease-activated receptor agonists

Protease-activated receptor (PAR)1 (SFLLRN) and PAR2 (SLIGKV) lyophilised agonist peptides (JPT, Berlin, Germany) were diluted in RPMI 1640 and added to cells (500 μM) overnight (37°C). Cells were incubated (15 min at room temperature) with 25 μg·mL^{-1} anti-PAR2 antibody (Santa Cruz Biotech) to block PAR2 agonists.

Statistics

Cytokine and proliferation data are presented as mean±SD and immunoblot analyses are presented as mean±SD after densitometric image analysis (ImageJ) of independent experiments. Paired or unpaired t-tests were performed and p-values <0.05 were considered significant.

RESULTS

HDM extract dose-dependently induced the release of IL-6 and increased the proliferation of BSM cells of asthma patients

BSM cells of asthma patients (n=5) and controls (n=5) were incubated with HDM extract for 24 h in the absence of FCS. HDM dose-dependently increased IL-6 release in both groups. A statistically significant increase in IL-6 ($p<0.05$) was observed only in BSM cells from asthma patients (fig. 1a). A significantly increased, dose-dependent proliferation in response to HDM extract was observed only in BSM cells from asthma patients (fig. 1b). As shown in figure 1c, HDM extract did not induce LDH release, and therefore had no cytotoxic effect.

HDM extract downregulated C/EBPα expression in BSM cells of asthma patients

HDM-extract (10 μg·mL^{-1}) significantly downregulated the expression of C/EBPα in BSM cells of asthma patients, but not in cells of controls ($p<0.05$; fig. 2a–d). Although the degree of the C/EBPα suppression was individual (range 50–100%), it was consistent in all experiments (n=6) (fig. 2a and b). In contrast, HDM extract incubation of BSM cells from nonasthmatic controls (n=7) did not significantly affect the expression of C/EBPα (fig. 2c and d). We used primary lung fibroblasts (n=2) to examine the cell specificity of the responses; these were incubated with HDM extract (10 μg·mL^{-1}), which did not have any effect on the expression of C/EBPα proteins (fig. 2e).

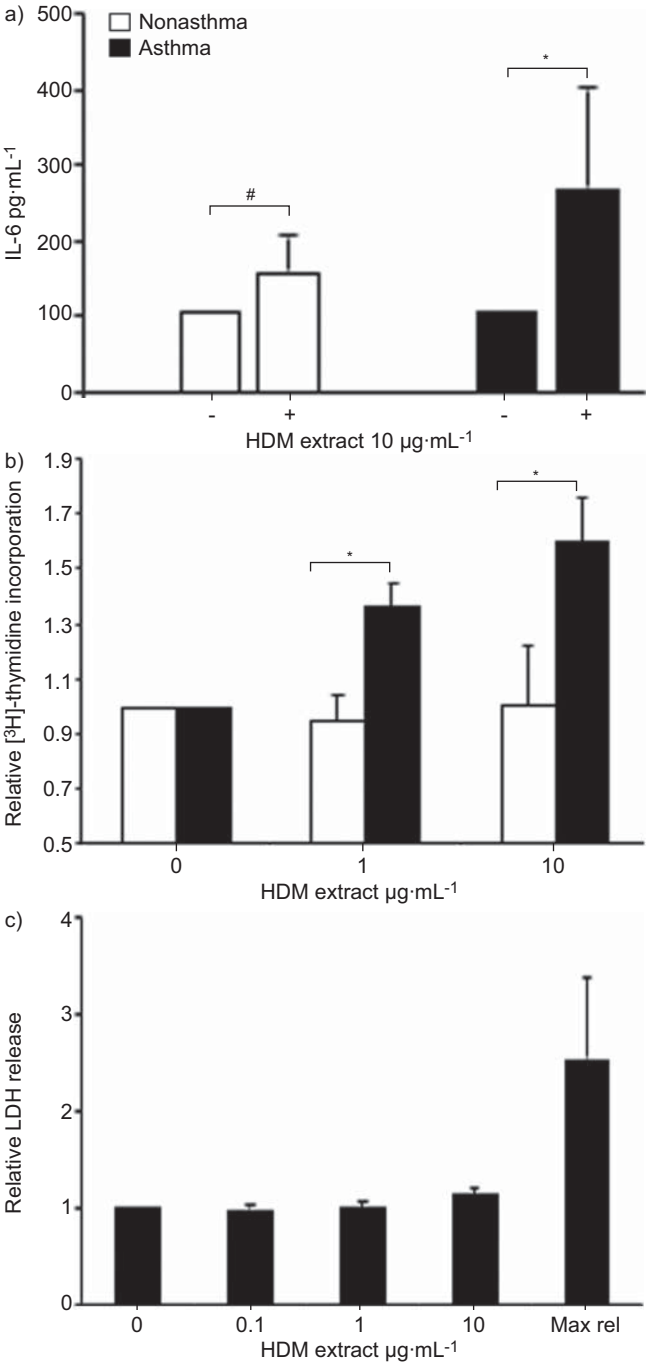


FIGURE 1. Pro-inflammatory and proliferative characteristics of bronchial smooth muscle (BSM) cells after house dust mite (HDM) extract treatment. a) HDM extract significantly increased interleukin (IL)-6 secretion after 24 h in BSM cells of asthmatic patients (n=5). b) Proliferation of BSM cells of asthmatic patients significantly increased in response to HDM extract after 48 h (nonasthmatic, n=4; asthmatic, n=4). c) Lactate dehydrogenase (LDH) release after 24 h incubation with HDM extract at 10 μg·mL^{-1} (n=3). Max rel: maximum release. #: nonsignificant; *: $p<0.05$.

HDM extract did not affect the reinitiation of 5′TOP mRNA translation and had no effect on eIFE4 expression

The reinitiation of 5′TOP mRNA *CEBPA* translation was monitored by TCRS, the principle of which is depicted

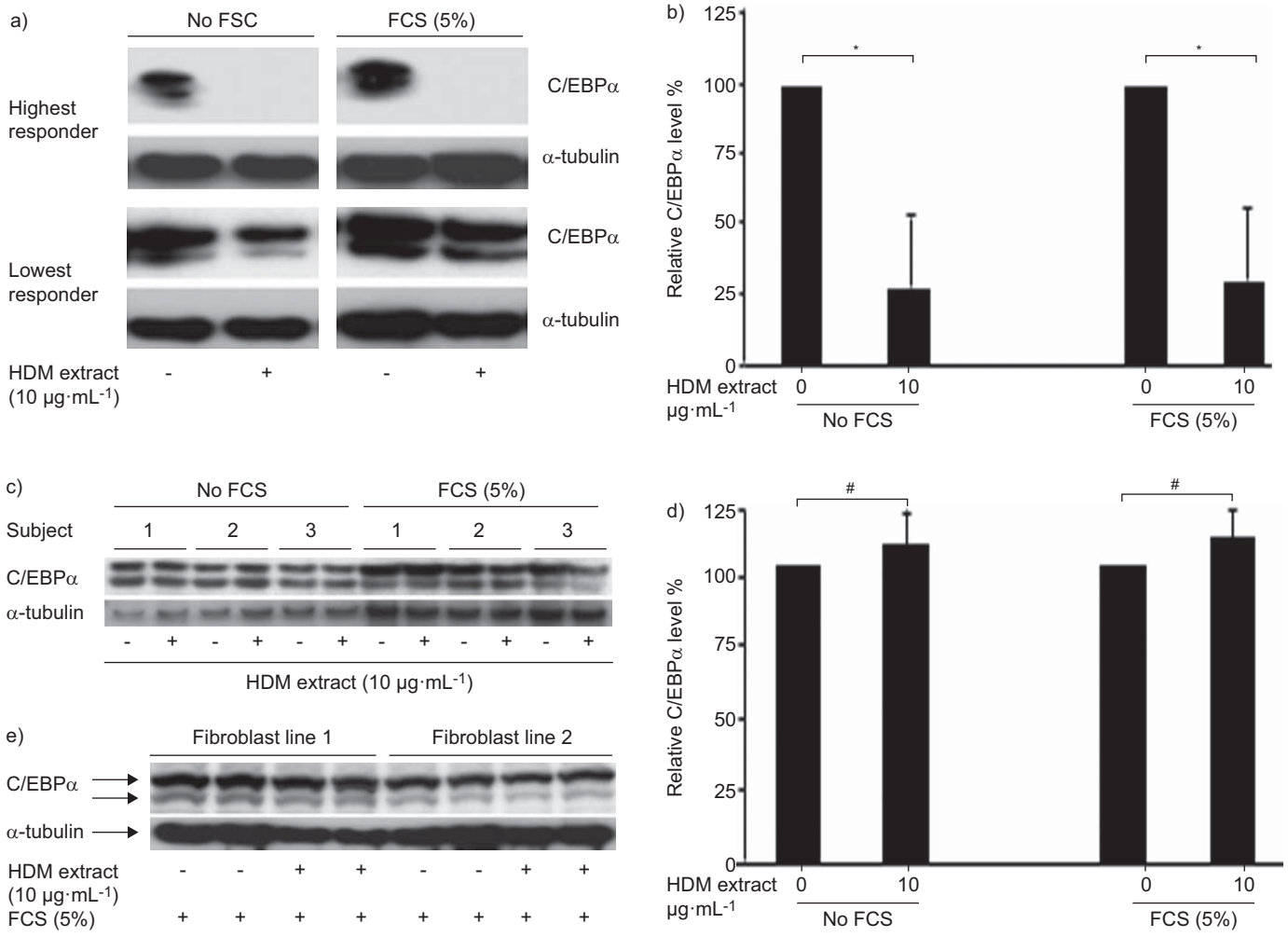


FIGURE 2. Immunoblot analysis of CCAAT/enhancer-binding protein (C/EBP)α expression after house dust mite (HDM) extract treatment in the absence or presence of fetal calf serum (FCS). a) Two representative immunoblots of two different bronchial smooth muscle (BSM) cells of asthmatic patients incubated for 24 h in 10 μg·mL^{-1} HDM extract. b) Densitometric analysis of immunoblots obtained in BSM cells of asthmatic patients (n=6). c) Representative immunoblot of BSM cells of nonasthmatic controls (n=3) incubated for 24 h in 10 μg·mL^{-1} HDM extract. d) Densitometric analysis of the data obtained from nonasthma patients (n=7). e) Representative immunoblot of primary lung fibroblasts (n=2) incubated for 24 h in 10 μg·mL^{-1} HDM extract. Duplicate of two independent experiments. #: nonsignificant; *: $p<0.05$.

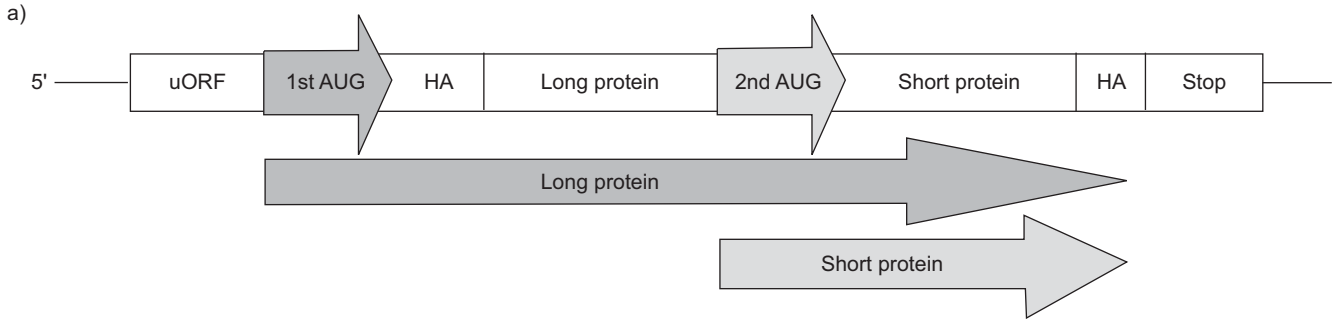
in figure 3a. The construct generates a long (p23) and a short (p12) peptide, of which the ratio (p12/p23) is a measure for translation reinitiation [14]. As shown in figure 3b, HDM extract did not significantly change the p12/p23 ratio, indicating that the reinitiation of translation of 5′TOP mRNAs was not affected by HDM extract. Translation of *CEBPA* mRNA is regulated by several proteins, including eIF4E, hnRNPE2 and CRT (fig. 3c). Consistent with the unchanged p12/p23 ratio, the expression of eIF4E in asthmatic cells (n=3) was unaffected after incubation with HDM extract (10 μg·mL^{-1}), indicating that a different mechanism controls translation (fig. 3d).

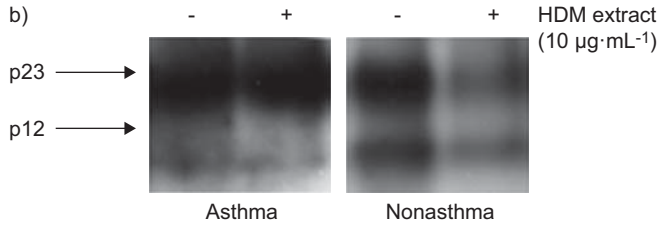
HDM extract upregulated the expression of CRT in BSM cells of asthma patients

HDM extract (10 μg·mL^{-1}) significantly increased the relative expression of CRT in BSM cells of asthma patients (n=5) in a time-dependent manner, but not in control cells (n=5). A significant increase ($p<0.05$) of CRT was detected after 60 min incubation with HDM extract in asthma BSM cells, compared with time-point 0 (fig. 4a), whereas the CRT level in non-asthma control cells was not significantly affected (fig. 4a). The specificity of this finding was assessed by incubating BSM cells with CRT-specific siRNA, which revealed an inverse relationship between the expression of CRT and that of C/EBPα protein (fig. 4b).

HDM extract-induced, protease-dependent morphological changes in BSM cells

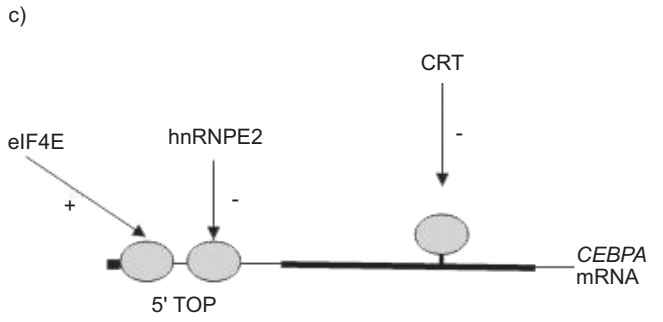
HDM extract exhibits high levels of proteolytic activity. Therefore, the effect on cell desquamation, and the involvement of PAR1 and PAR2, were assessed. HDM extract (10 μg·mL^{-1}) induced morphological changes in BSM cells within 24 h (fig. 5a). A partial detachment of BSM cells was observed in the presence of HDM extract (10 μg·mL^{-1}), but this was insufficient to induce a complete cell desquamation. In the presence of 5% FCS, the BSM cell detachment was not observed, indicating the involvement of a protease-dependent





p12/p23

	Control	HDM extract		Control	HDM extract
NA1	0.98	0.94	A1	0.69	0.80
NA2	0.64	0.88	A2	0.21	0.19
NA3	0.69	0.63	A3	0.76	0.75
Mean	0.77	0.82	Mean	0.55	0.58
SEM	0.18	0.16	SEM	0.30	0.34



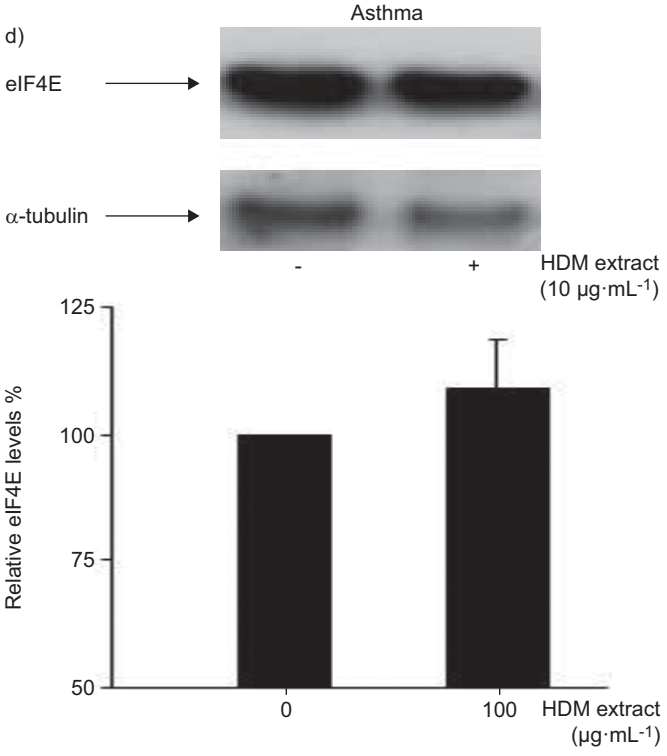


FIGURE 3. Immunoblot analysis of translation control of CCAAT/enhancer-binding protein (C/EBP)α. a) Schematic representation of the translation control reporter system. Impaired translation leads to lower reinitiation at the second start codon (AUG) and, as a consequence, to decreased expression of the short protein (p12). b) Representative immunoblot of the expression of long (p23) and short proteins in bronchial smooth muscle (BSM) cells of an asthmatic patient and a nonasthmatic control, and calculated p12/p23 ratios (asthma (A), n=3; non-asthma (NA), n=3). c) Schematic representation of regulatory proteins and their docking sites of the translation mechanisms for C/EBPα. d) Representative immunoblot of the expression level of eukaryotic initiation factor (eIF)4E in BSM cells of an asthma patient incubated for 24 h with 10 μg·mL^{-1} house dust mite (HDM) extract, and densitometric analysis of immunoblots obtained in BSM cells of asthmatic patients (n=3). uORF: upstream open reading frame; HA: haemagglutinin epitope; hnRNP: heterogeneous nuclear ribonucleoprotein; CRT: calreticulin.

mechanism. Therefore, the effect of HDM extract, and PAR1 and PAR2 agonists, on the expression of C/EBPα protein was assessed in BSM cells of asthmatics and controls (n=5). In BSM cells of asthma patients, the expression of C/EBPα protein in response to 24 h treatment with HDM extract or a PAR2 agonist was dramatically reduced, whereas the PAR1 agonist had no effect (fig. 5b and c). The involvement of PAR2 in the HDM extract-induced downregulation of C/EBPα was completely reversed by a specific PAR2-blocking antibody (fig. 5b and c).

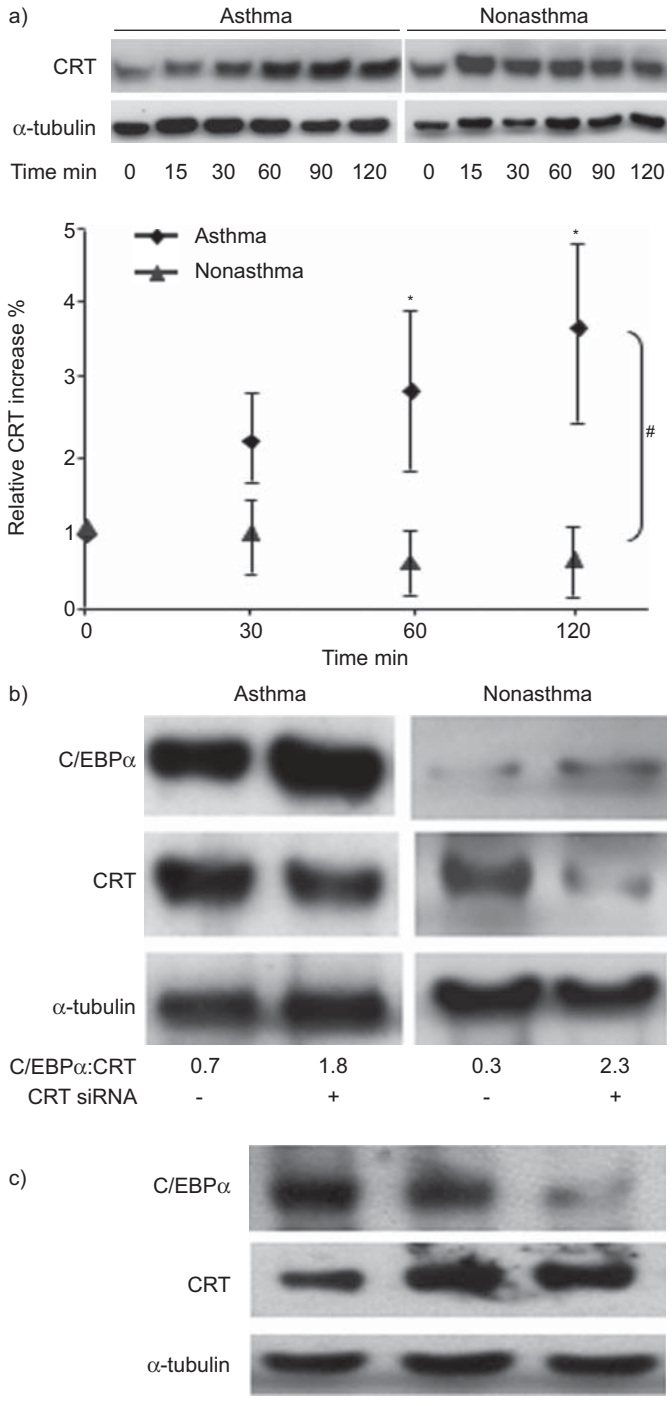


FIGURE 4. Immunoblot analysis of calreticulin (CRT) expression after house dust mite (HDM) extract treatment. a) Time-dependent expression of CRT in bronchial smooth muscle (BSM) cells after HDM extract treatment and the calculated relative increase in BSM cells of asthma patients (n=5) *versus* BSM cells of nonasthma controls (n=5). b) CCAAT/enhancer-binding protein (CEBP)α expression in BSM cells of asthmatic and nonasthmatic subjects after transient knockout of the CRT by siRNA and calculated C/EBPα:CRT ratio. Data shown are representative for four independent experiments. c) Inverse correlation between expression levels of CRT and C/EBPα. *: $p<0.05$ for asthma BSM cells at 0 *versus* 60 and 120 min; #: $p<0.05$ for asthma *versus* nonasthma BSM cells at 120 min.

Figure 5d demonstrates that HDM extract specifically downregulated smooth muscle α-actin in BSM cells obtained from asthma patients (n=2) and this result coincided with the aforementioned diminished levels of C/EBPα.

DISCUSSION

In the present study, we showed that HDM extract downregulated C/EBPα protein expression in BSM cells of asthma patients, but not in BSM cells and fibroblasts of nonasthmatic subjects. The downregulation was mediated *via* two mechanisms, which were independent of the immune system.

The first mechanism was mediated *via* CRT; the second involved PAR2. These findings further substantiate the importance of C/EBPα translation in asthma pathology and how BSM cell hyperplasia may be triggered by external stimuli, in particular by HDM. Together with our previous findings, which showed diminished C/EBPα protein levels associated with increased proliferative capacity of BSM cell of asthma patients, this may explain the increased bulk of smooth muscle cells as found in the airways of asthma patients [13, 14, 20]. In addition, in response to HDM extract, BSM cells of asthma patients showed increased proliferation and IL-6 secretion. Thus, HDM has the capacity to elicit an inflammatory response and induce airway remodelling as the result of a direct action on resident cells of the lung, independent of the immunological compartment. Of course, we realise that the HDM immunoglobulin (Ig)E-driven immune response is of great importance to understanding atopic asthma, but it should also be realised that not all asthma is associated with IgE, in particular intrinsic asthma [21].

Previously, we found that an impaired initiation of the translation of *CEBPA* mRNA in BSM cells of asthma patients was associated with the decreased expression of the translation regulator eIF4E [14]. Here, we could not detect an impaired translation using the translation reporter construct [22, 23], indicating that a different mechanism may be involved. We proposed that CRT, a protein initially identified as an endoplasmic reticulum luminal chaperone that controls the regulation of intracellular Ca^{2+} homeostasis [24], could be pivotal in the HDM-induced downregulation of C/EBPα. It was recently shown that binding of CRT inhibited the translation of the *CEBPA* mRNA, as a result of a direct interaction of CRT and the *CEBPA* transcript. CRT was shown to bind the stem loop within the *CEBPA* mRNA, which is formed by internal base-pairing of the GCN repeat motif [17]. This loop then functions as the docking site for CRT and prevents the translation of *CEBPA* mRNA into protein. An inverse relationship of C/EBPα and CRT had been demonstrated in adipocytes, where CRT inhibited adipogenesis by repressing the expression of C/EBPα [25], an observation that was also reported in acute myeloid leukaemia [26]. Here, we demonstrated that the same mechanism operates in BSM cells of asthma patients, as transient suppression of CRT by siRNA restored C/EBPα levels. Therefore, we propose that the decrease of the C/EBPα protein level in BSM cells of asthma patients may be related to HDM extract-induced sequestration of the corresponding mRNA by CRT. Since FCS is a strong inhibitor of protease activity, we infer that HDM-induced CRT expression is independent of PAR activation.

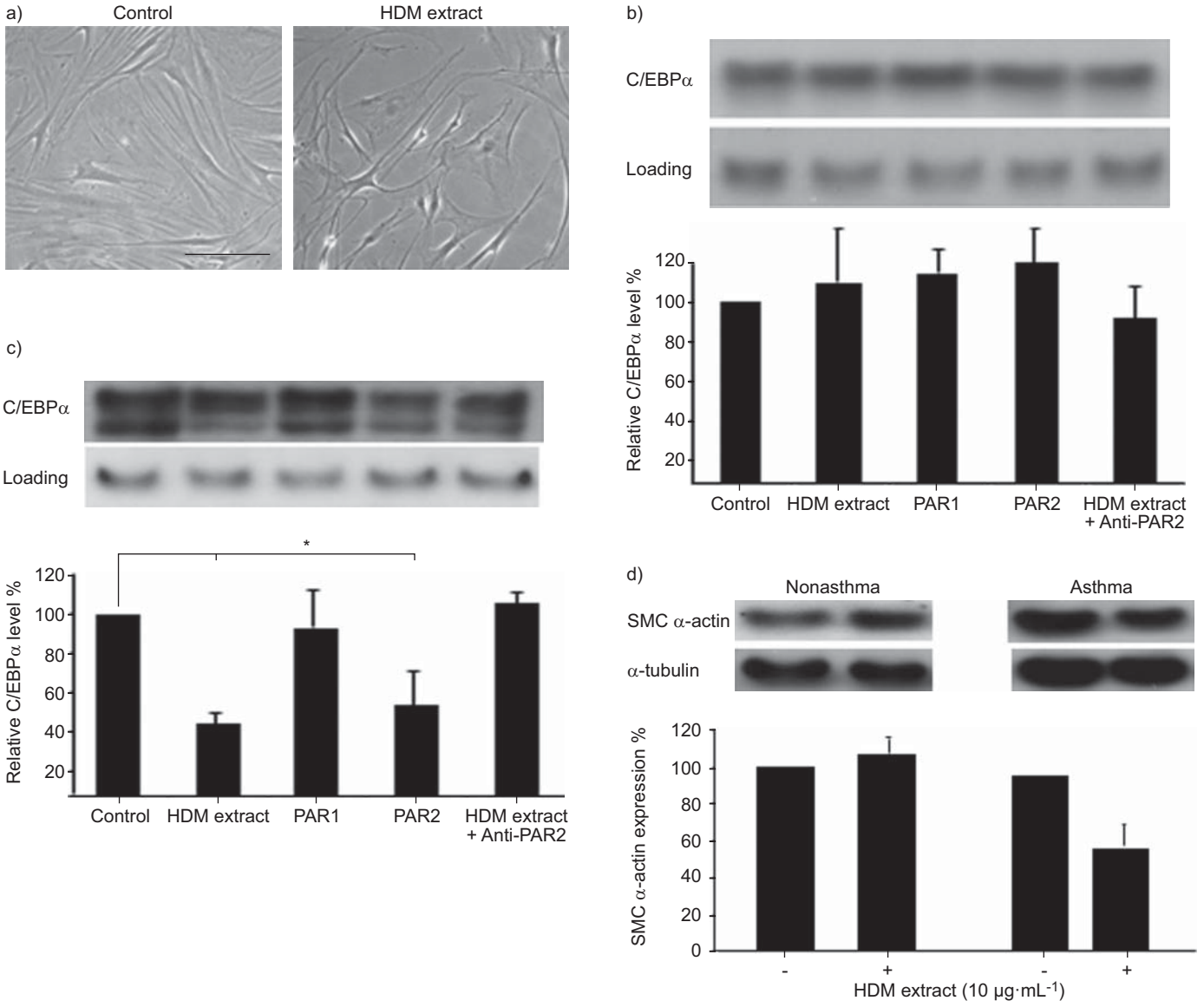


FIGURE 5. Morphological changes of bronchial smooth muscle (BSM) cells after treatment with house dust mite (HDM) extract, and immunoblot analysis of CCAAT/enhancer-binding protein (C/EBP)α expression after incubation with protease-activated receptor (PAR)1 and PAR2 agonists. a) HDM extract-induced cell–cell detachment in BSM cells after 24 h incubation. Scale bar=100 μm. C/EBPα expression in BSM cells of b) a nonasthmatic control and c) an asthma patient after 24 h incubation with HDM extract, PAR1 agonist, PAR2 agonist, or HDM extract together with PAR-2 blocking antibodies (anti-PAR2). Data shown are representative of five independently performed experiments. d) Smooth muscle cell (SMC) α-actin expression in BSM cells of a nonasthmatic control (n=3) and an asthma patient (n=2) after 24 h incubation with HDM extract. *: $p<0.05$.

HDM allergens are omnipresent and by far the most important indoor IgE-triggering compound [7], and have a potent pro-inflammatory and desquamating effect on airway epithelial cells [11]. Here, we also provide evidence that HDM extract affected the behaviour of BSM cells of asthma patients by downregulating *CEBPA* gene expression, which involved a mechanism using PAR2. Our data show that a PAR2 agonist decreased C/EBPα expression in BSM cells of asthma patients, but not in controls. Hindering the access of HDM allergens to PAR2 with blocking antibodies counteracted this downregulation of C/EBPα. In line with this finding, the HDM allergens Der p1 and Der p5 activated human airway-derived epithelial cells by a protease-dependent and -independent mechanism [11]. In the present study, similar protease-dependent and -independent mechanisms may be involved, because in the presence of FCS, which is a potent protease activity blocker, HDM allergens still partially downregulated C/EBPα proteins. This suggested that the pathway involving CRT is independent of the activation of PARs. In addition, the reduced expression of C/EBPα may also lead to lower levels of smooth muscle α-actin, because the expression of the gene is controlled by C/EBP isoforms [27, 28]. It should be emphasised, however, that our observations were performed on a small group of untreated mild and medication-treated moderate–severe asthma patients.

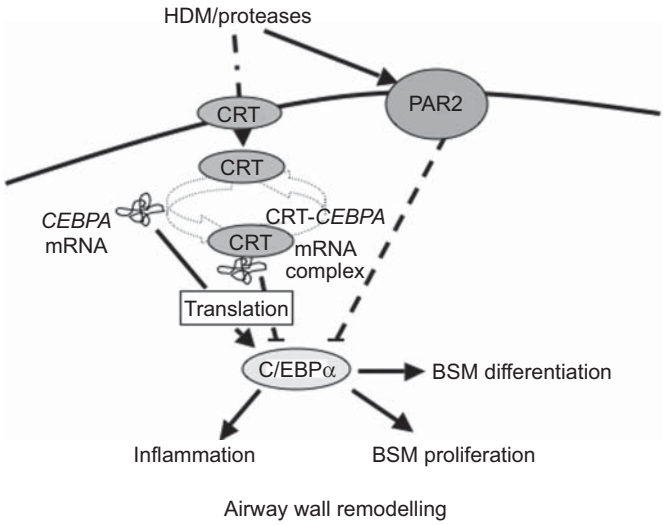


FIGURE 6. Schematic overview of distinct activation mechanisms triggered by house dust mite (HDM) extract that affect the expression of CCAAT/enhancer-binding protein (C/EBP)α in bronchial smooth muscle (BSM) cells. CRT: calreticulin; PAR: protease-activated receptor.

Future studies in three well-defined asthma patients groups (mild, moderate, severe and treated *versus* untreated) are planned to further substantiate the importance of these signalling pathways, and whether treatment affects them.

Taken together, these results indicate that the HDM-induced downregulation of C/EBPα is specific for BSM cells of asthma patients and involves PAR-dependent and -independent mechanisms, the latter mechanism involving an induction of CRT.

The downregulation of C/EBPα in BSM cells by HDM extract may be a first indication that there is a link between the pathologies in atopic and nonatopic asthma at the level of the smooth muscle cell. Hyperplasia of BSM cells in the bronchi of asthmatic patients might result from repressed translation of the *CEBPA* mRNA as a result of ongoing exposure to allergens. An integrated, schematic overview of our ideas on protease-dependent and -independent mechanisms, and how they are involved in airway wall remodelling is shown in figure 6. In the light of our present findings, new C/EBPα-tailored asthma therapies that directly target the resident cells of the airway wall could be envisaged.

SUPPORT STATEMENT

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STATEMENT OF INTEREST

A statement of interest for this study can be found at www.erj.ersjournals.com/site/misc/statements.xhtml

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