

Original article

Variable p-CREB expression depicts different asthma phenotypes

Background: Chromatin modification may play a role in inflammatory gene regulation in asthma. Cyclic adenosine mono-phosphate response element-binding protein (CREB), with the specific co-activator, the CREB-binding protein (CBP), contributes to the acetylation of chromatin and to the transcription of pro-inflammatory genes.

Objectives: To evaluate the expression of CBP and of phospho-CREB (p-CREB) in bronchial biopsies and in peripheral blood mononuclear cells (PBMC) of controls (C), untreated (UA), inhaled steroid treated (ICS) and steroid-dependent asthmatic (SDA) patients.

Methods: We used immunohistochemistry in bronchial biopsies and western blot analysis and immunocytochemistry in PBMC.

Results: Cyclic adenosine mono-phosphate response element-binding protein expression, in the epithelium was similar in all groups, while p-CREB expression was increased in UA and in SDA in comparison with ICS and C subjects (C vs UA $P = 0.002$, C vs SDA $P = 0.007$), (ICS vs SDA $P = 0.005$), (ICS vs UA $P = 0.001$). Interestingly, also in the submucosa, p-CREB was increased in UA and SDA in comparison with ICS and C subjects (C vs UA $P = 0.0004$) (C vs SDA $P < 0.0001$) (ICS vs UA $P = 0.002$) (ICS vs SDA $P < 0.0001$) and positively correlated with leukocyte infiltration within the bronchi (CD45RB+ cells). Similar results were obtained with PBMC isolated from the same patient groups. Incubation of PBMC *in vitro*, with fluticasone propionate, decreased the p-CREB expression induced by cytokine activation (interferon- γ , tumor necrosis factor- α).

Conclusions: This study demonstrates that the expression of p-CREB is related, in asthma, to the persistent inflammation according to the disease severity. p-CREB expression can be modulated by glucocorticoids in responsive patients.

G. Chiappara¹, P. Chanez², A. Bruno¹,
E. Pace¹, F. Pompeo¹, J. Bousquet²,
G. Bonsignore¹, M. Gjomarkaj¹

¹Istituto di Biomedicina e Immunologia Molecolare, Comessa di Immunopatologia e Farmacologia Sperimentale dell'Apparato Respiratorio, Consiglio Nazionale delle Ricerche, Palermo, Italy; ²Clinique des Maladies Respiratoires, INSERM-U454, CHU Montpellier, France

Key words: asthma; glucocorticoids; inflammation.

Giuseppina Chiappara, PhD
Istituto di Biomedicina e Immunologia Molecolare
Consiglio Nazionale delle Ricerche
Via Ugo La Malfa
153 – 90146 Palermo
Italy

Accepted for publication 6 April 2007

Introduction

Asthma is a chronic inflammatory disease characterized by increased expression of multiple inflammatory genes. Chromatin modification plays a critical role in the regulation of these genes. Acetylation of histones by acetyltransferases (HATs) is associated with increased gene transcription. Among these mechanisms, the activity and the availability of HAT is important because it is able, via the histone acetylation, to switch-on pro-inflammatory genes (1, 2). This effect is achieved via the interaction of specific transcription factors, including the nuclear factor κ B (NF κ B) (3) and the cyclic adenosine mono-phosphate response element-binding protein (CREB), with the specific co-activator, the CREB-binding protein (CBP) (4). This interaction leads, in turn, to the acetylation of chromatin and to the consequent transcription of pro-inflammatory genes (5).

The use of inhaled glucocorticoids represents the cornerstone treatment for asthma because of their broad range of anti-inflammatory activities and to the good safety profile. At the nuclear level, the complex glucocorticoids bound to its receptor (GR) increases the transcription of anti-inflammatory genes by interacting with co-activator molecules, including CBP (3, 6) and thus switching-on histone acetylation and gene transcription of anti-inflammatory genes (1). Moreover, the steroid-GR complex exerts its anti-inflammatory action by down-regulating NF κ B (3), leading, in turn, to a reduced acetylation and transcription of pro-inflammatory genes (7). In this scenario, it is not clear whether the phosphorylation of CREB is affected by steroids.

Most of the patients treated with this method have seen their asthma controlled. A subset of patients remained uncontrolled despite the increase in the use of various drugs, including high doses of inhaled glucocorticoids,

long-acting β_2 agonists and a continuous dose of oral glucocorticoids. These severe patients have persistent ongoing local and systemic inflammation with an attenuated response to the current anti-inflammatory treatment with steroids (8).

Although the bronchial epithelium is considered the typical target of glucocorticoids in asthma (9), it is also known that this disease involves other districts, including the bronchial submucosa and that the effects of glucocorticoids are also exerted at this level (10, 11). Moreover, it has been also demonstrated that asthma can be considered as a 'systemic' disease and, accordingly, it is characterized by a persistent peripheral T-cell activation (10).

We aimed to examine in the present study the persistence of pro-inflammatory factors, such as phospho-CREB (p-CREB), as the key feature in asthma and its variability in the epithelium, in infiltrating bronchial leukocytes and in peripheral blood. The potential for glucocorticoids to modulate this expression, in different clinical and *in vitro* situations, was then considered.

Materials and methods

Study population

Thirty-one asthmatic subjects were selected according to the American Thoracic Society's criteria, as previously described (12). The clinical severity of asthma was assessed by the Global Initiative for Asthma guidelines (8). Subjects were divided into three groups according to the treatment. We assessed the airway hyper-responsiveness by methacholine challenge in mild asthmatics with a forced expiratory volume in 1 s $> 70\%$ of predicted values and not in severe asthmatics for safety reasons following the European Respiratory Society (ERS) Guidelines. A positive methacholine challenge < 16 mg/ml (PC20) was shown in the tested patients. The first group included 11 untreated asthmatics (UA) with mild intermittent asthma who received inhaled short-acting β_2 -agonists, as needed. The second group included nine inhaled steroid treated asthmatics (ICS) with severe asthma who required to be controlled daily with a high dose of inhaled corticosteroids (3000 μ g of beclomethasone dipropionate equivalent), long-acting β_2 -agonists (100 μ g salmeterol), and inhaled short-acting β_2 -agonists, as required. The third group of 11 cortico-steroid-dependent asthmatics (SDA) was defined, as previously described (13). These subjects had severe persistent asthma that required a daily dose of ICS (3000 μ g of beclomethasone dipropionate equivalent) and an oral dose of prednisone, long-acting β_2 -agonists (100 μ g salmeterol), and inhaled short-acting β_2 -agonists as required. Nine healthy subjects were included as controls. All patients were nonsmokers. Subjects who had any bronchial or respiratory tract infection during the month preceding the test, were excluded from the study.

This study was approved by our ethics committee and the patients did sign a written consent.

Enumeration of CBP+, p-CREB+, and CD45RB+ cells in bronchial biopsies

Four-to-six bronchial biopsies were recovered, fixed and processed for immunohistochemistry, as previously described (13, 14). Specimens were incubated with the following antibodies: a rabbit

polyclonal anti-CBP-NT (15) (Upstate Biotechnology, Lake Placid, NY, USA). (1 : 40 dilution, 1 h, room temperature), a rabbit polyclonal anti-p-CREB (Upstate Biotechnology) (heat pretreatment at 92°C; 1 : 40 dilution; overnight incubation) (16) and a mouse monoclonal antibody anti-human CD 45RB, Clone PD7/26 diluted 1 : 50 (1 h, room temperature) with a pretreatment (trypsin 0.1%, 37°C, 10 min) (17).

The reaction was revealed by using the avidin-biotin technique (AP-LSAB2 KIT DAKO, Glostrup, Denmark). Control slides were made by using an antibody diluent (DAKO) and irrelevant rabbit or mouse antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) of the same isotype and at the same concentration of the primary antibodies. The cell nuclei were stained for 1 min with hematoxylin (DAKO).

The immunoreactivity of each studied marker in the submucosa and in the epithelium was evaluated blindly by two independent investigators (GC and AB). Slides were evaluated using a Leica (Wetzlar, Germany) microscope $\times 400$ magnification. In the submucosa, the number of positive cells per mm² was evaluated, as previously described (13, 14). p-CREB and CD45RB immunoreactivity was evaluated in serial sections and in the same region of the biopsy of the same patients. In the epithelium, positive cells were evaluated per area over a minimum length of 260 mm from the internal side of the epithelium (18). The Image was then subsequently analyzed using a Quantimet 500 MC software (Leica) for image analysis. The results are expressed as the medians and 25–75 percentiles of the total measurements (at least four measurements were performed to calculate averages).

Western blot analysis of p-CREB expression in PBMCs

Peripheral blood mononuclear cells (PBMC) cells from C, UA, ICS, and SDA subjects were freshly isolated by the Ficoll-Hypaque gradient and the western blot analysis for p-CREB was performed as previously described (19). Five micrograms of total protein were loaded in the gel. All blots were first probed using a rabbit polyclonal anti-p-CREB (Upstate Biotechnology) diluted 1 : 100 and, after stripping procedures, were probed using an anti-CREB (Upstate Biotechnology) to detect total amounts of the same protein. Revelation was performed with an enhanced chemiluminescence system (NEN, Boston, MA, USA) followed by autoradiography (19). Lysates of mouse 3T3 cells treated with forskolin formulated in phosphate buffer saline/bovine serum albumin (BSA) were used as positive controls (data not shown). Negative controls were performed in the absence of primary antibody or including an isotype control antibody (data not shown). Data were expressed as the ratio of the band intensity of the phosphorylated protein versus the band intensity of the total protein. Beta-actin (Sigma) was used as the housekeeping protein to normalize differences in protein loading.

Enumeration of p-CREB+ in PBMC

Isolated PBMC were evaluated for p-CREB expression by immunocytochemistry using a rabbit polyclonal anti-p-CREB (Upstate Biotechnology) (1 : 5 dilution; overnight incubation). The reaction was revealed by using the avidin-biotin technique (AP-LSAB2 KIT DAKO). Results were expressed as percentages of positive cells.

Western blot analysis of fluticasone propionate modulation of p-CREB expression in PBMC stimulated with IFN γ and/or TNF α

Freshly isolated PBMC from normal subjects were incubated for 30 min with interferon- γ (IFN γ) gamma (100 ng/ml) and tumor

necrosis factor- α (TNF α) alpha (10 ng/ml) (20, 21), either alone or in combination in the presence and absence of fluticasone propionate (FP) 10^{-7} M. PBMC treated with LPS (100 ng/ml) were used as positive controls (21). Western blots were performed as above described (19).

Statistical analysis

Data were analyzed using the Kruskal–Wallis test and Bonferroni Dunn's *post hoc* test. $P < 0.05$ was considered as statistically significant. Results are expressed as medians and 25th–75th percentiles.

Results

Demographic characteristics of the subjects

The demographic characteristics as well as the differences in airflow obstruction of the four study groups are shown in Table 1.

Immunoreactivity of p-CREB in bronchial epithelium

We first evaluated the expression of p-CREB in bronchial epithelium. The p-CREB immunoreactivity was significantly increased in UA and in SDA in comparison with ICS and control subjects (Fig. 1). The immunoreactivity was mainly present in the basal epithelial cells rather than in columnar epithelial cells and was concentrated in the nucleus and in the cytoplasm of the cells. No concentration of p-CREB immunoreactivity was specifically detected in the areas with epithelial shedding.

Immunoreactivity of p-CREB in bronchial submucosa

In the submucosa, p-CREB immunoreactivity was increased in UA and SDA in comparison with ICS and C subjects (Fig. 2). We did not observe any extracellular matrix deposit or any immunoreactivity at the level of smooth muscle and of the vessels when they were present in the biopsies.

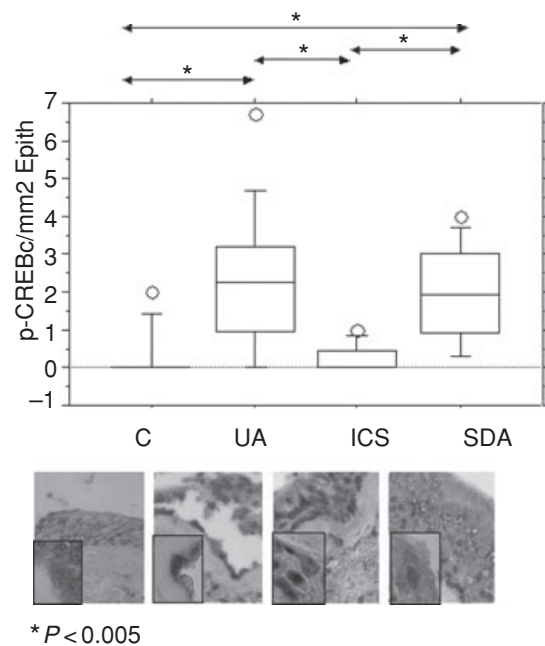


Figure 1. Expression of phospho-cyclic adenosine monophosphate response element-binding protein (p-CREB) in bronchial epithelium. Sections from control (C) ($n = 9$), untreated asthmatic (UA) ($n = 11$), inhaled steroid treated (ICS) ($n = 9$), and steroid-dependent asthmatic (SDA) ($n = 11$) were prepared as described in Materials and methods. Results are expressed as number of cells per mm^2 . Results are expressed as medians and 25th–75th percentiles. Representative images are at $40\times$ magnification. Lower left corner, representative images at $100\times$.

Immunoreactivity of CBP in bronchial epithelium and bronchial submucosa

We then evaluated the expression of CBP in bronchial epithelium as well as in bronchial submucosa. The expression of CBP, in the two compartments, was similar in the four study groups (Table 2).

Table 1. Demographic characteristics of the subjects

Characteristics	Asthmatic subjects group			
	C	UA	ICS	SDA
No. of subjects	9	11	9	11
Age (years)	29.5 (25–34)	21 (20–52)	46 (30–51)	53 (44–61)
Sex (% male)	62	63	46	63
FEV1 (% predicted)	108.5 (103–113.5)	100 (97–111.5)	60 (53.7–66)	50 (40–75.5)
Oral prednisone dose (mg/day)	0	0	0	30 (21.2–37.5)
Allergy (%)	0	81.8	77.7	63.6

Results are expressed in medians; 25th–75th percentile values are shown in parenthesis. C, normal; UA, untreated asthma; ICS, inhaled corticosteroids; SDA, oral corticosteroids dependent; FEV1, forced expiratory volume in 1 s.

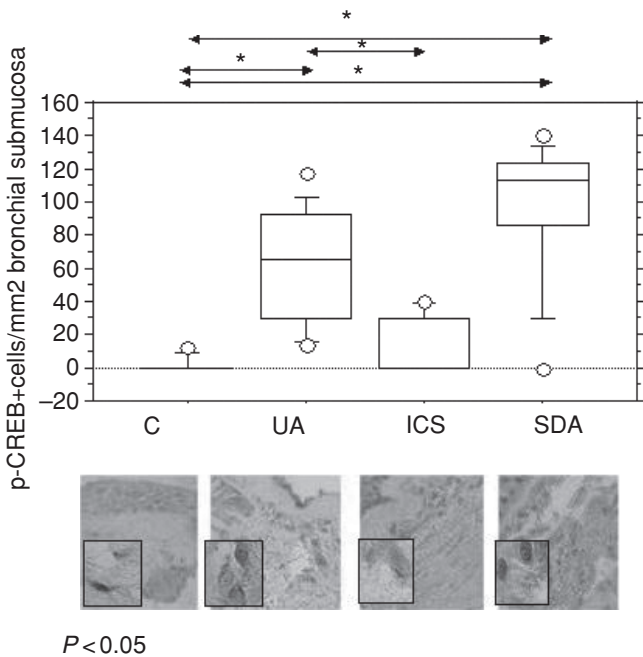


Figure 2. Expression of phospho-cyclic adenosine mono-phosphate response element-binding protein (p-CREB) in bronchial submucosa. Sections from control (C) ($n = 9$), untreated asthmatic (UA) ($n = 11$), inhaled steroid treated (ICS) ($n = 9$), and steroid-dependent asthmatic (SDA) ($n = 11$) were prepared as described in Materials and methods. Results are expressed as number of cells per mm^2 . Results are expressed as medians and 25th–75th percentiles. Representative images are at 40 $\times$ magnification. Lower left corner, representative images at 100 $\times$.

Correlation of p-CREB and CD45RB positive cells in bronchial submucosa

We found a positive correlation between p-CREB expression and the number of CD45RB positive cells in the submucosa in all the study groups (Fig. 3).

Expression of p-CREB in PBMCs

The expression of p-CREB was evaluated by western blot analysis and by immunocytochemistry in PBMC. The expression of p-CREB and the p-CREB/CREB ratio was higher in SDA and UA when compared with ICS and C (Fig. 4).

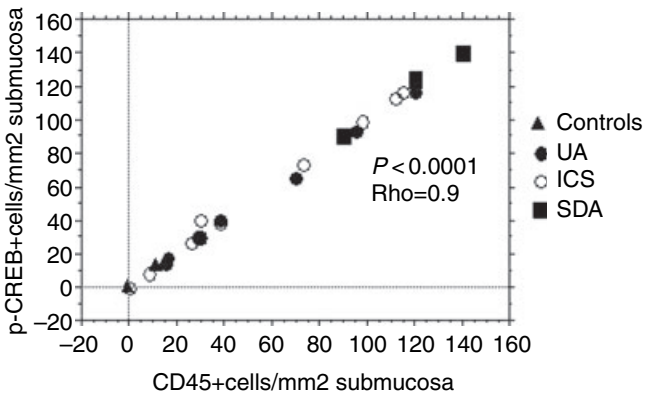


Figure 3. Correlation between phospho-cyclic adenosine mono-phosphate response element-binding protein (p-CREB) and CD45RB in bronchial submucosa of all studied subjects. Statistical analysis by Spearman rank correlation test.

Inhibition by fluticasone of $\text{IFN}\gamma$ and $\text{TNF}\alpha$ induced expression of p-CREB in normal PBMC

Interferon- γ and $\text{TNF}\alpha$, either alone or in combination, were able to significantly increase the p-CREB/CREB ratio in normal PBMC, while the addition of FP 10^{-7} M to the PBMC stimulated with the two pro-inflammatory molecules, either alone or in combination, was able to partially, but significantly, down-regulate the induced increase of p-CREB/CREB ratio (Fig. 5).

Discussion

Bronchial inflammation is the central feature of asthma, and is characterized by the recruitment and the activation of inflammatory cells within the airways. Here, we demonstrate that the expression of p-CREB, but not of CBP, is increased in the epithelium and in the submucosa of UA and SDA and correlates with the leukocyte infiltration of the submucosa. The increased p-CREB expression and increased p-CREB/CREB ratio were also present in PBMC from the same subjects indicating a systemic activation in asthma. Moreover, pro-inflammatory mediators involved in the pathogenesis of bronchial asthma, such as $\text{IFN}\gamma$ and $\text{TNF}\alpha$, were able to increase the p-CREB/CREB ratio and FP, a drug widely used in

Table 2. Cyclic adenosine mono-phosphate response element-binding protein expression in bronchial epithelium and in submucosa

Asthmatic subjects group	C	UA	ICS	SDA	P-value C vs UA	P-value C vs SDA	P-value C vs ICS	P-value ICS vs SDA	P-value ICS vs UA	P-value SDA vs UA
Epithelium	0.3 (0–2.3)	0.4 (0.2–2)	0.2 (0–0.4)	0.4 (0.2–0.5)	NS	NS	NS	NS	NS	NS
Submucosa	0.2 (0.10–3)	0.5 (0.25–0.6)	4.5 (0–10)	0.5 (0.3–3.2)	NS	NS	NS	NS	NS	NS

Results in medians and 25–75 percentiles. Statistical analysis by Kruskal–Wallis test and Bonferroni–Dunn's *post hoc* analysis. C, normal; UA, untreated asthma; ICS, inhaled corticosteroids; SDA, oral corticosteroids dependent; NS, not significant.

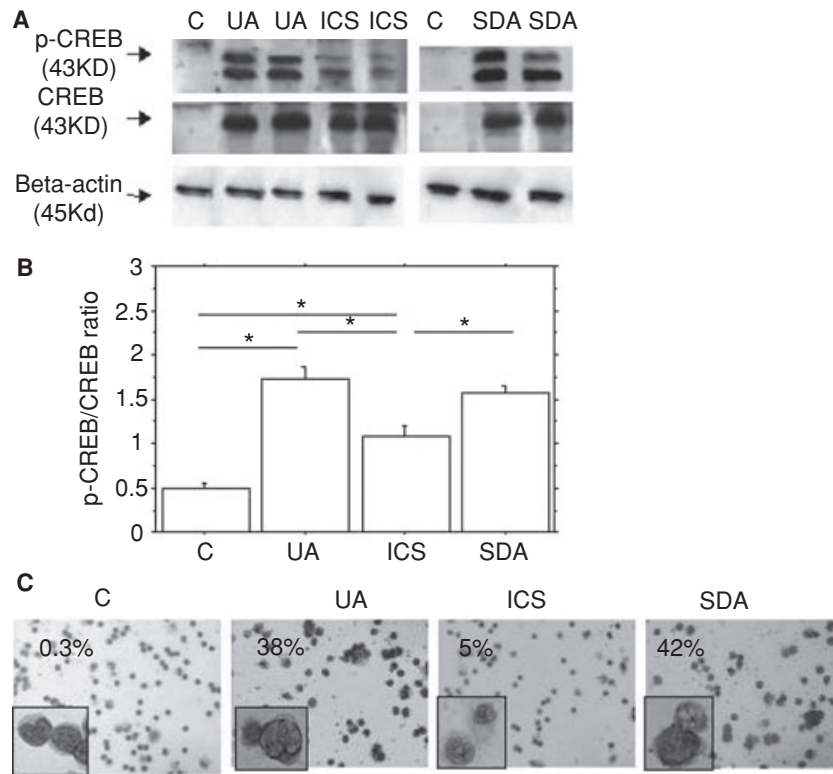


Figure 4. Expression of phospho-cyclic adenosine mono-phosphate response element-binding protein (p-CREB) in peripheral blood mononuclear cells (PBMC). PBMC were isolated from control (C) ($n = 5$), untreated asthmatic (UA) ($n = 5$), inhaled steroid treated (ICS) ($n = 5$), and steroid-dependent asthmatic (SDA) ($n = 5$) subjects and cells and protein extracts were analyzed for p-CREB expression by immunocytochemistry and by western blot analysis, respectively. (A) Representative western blot analysis of p-CREB in C, in UA, in ICS, and in SDA. The same blots were probed with monoclonal antibodies directed against p-CREB, CREB, and against β -actin. (B) Signals corresponding to p-CREB and CREB on the various western blots were semi-quantified by densitometric scanning, normalized for β -actin and results were expressed as p-CREB/CREB ratio; $*P < 0.05$. (C) Expression of p-CREB by immunocytochemistry. Results were expressed as percentage of positive cells. Representative images (20 $\times$) of p-CREB expression in PBMC of C, UA, ICS, and SDA. Lower left corner, Magnification 100 $\times$ of the cells.

the therapy of this disease, were able to revert this phenomenon *in vitro*.

It has been demonstrated that the complex and highly dynamic interactions between the bronchial epithelium and the submucosa and the consequent release of pro-inflammatory mediators are events crucially involved in the asthma pathogenesis (13, 14). The increased synthesis of pro-inflammatory mediators is the result of complex molecular mechanisms, whereby inflammatory genes are switched on by transcription factors and histone acetylation events. When pro-inflammatory transcription factors, including CREB, are activated via phosphorylation events, they bind specific DNA recognition sequences and interact with large co-activator molecules such as CBP. These co-activator molecules, having an intrinsic acetyl transferase activity, acetylate and open the chromatin structure, thus allowing inflammatory gene transcription (22). We evaluated the expression of CBP and, accordingly to a previous report (23), we showed that there is no difference in the expression of CBP among the four study groups. Conversely, we demonstrated that the p-CREB

expression was increased in the bronchial epithelium and in the submucosa of UA and SDA supporting the concept that this molecule was involved in the transcription of pro-inflammatory genes in bronchial asthma. In severe uncontrolled patients, the persistent inflammation is the hallmark of the decreased response to glucocorticoids.

The fact that this increase is not detected in the epithelium and in the submucosa of ICS suggests that, during efficient glucocorticoid treatment, the known down-regulation of the release of pro-inflammatory mediators might be, at least in part, related to the effects of glucocorticoids on the p-CREB expression.

Fragility and activation of bronchial epithelium are characteristics of asthma which are accompanied by a significant increase in apoptotic cells in asthmatic bronchial epithelium (24). The p-CREB is an important regulator of apoptosis in oxidant-mediated responses of lung epithelial cells as the reduction of p-CREB activity in epithelial cells exposed to H_2O_2 leads to a decrease in cell apoptosis together with enhanced *bcl-2* transcription (25). This concept is further supported by the finding that

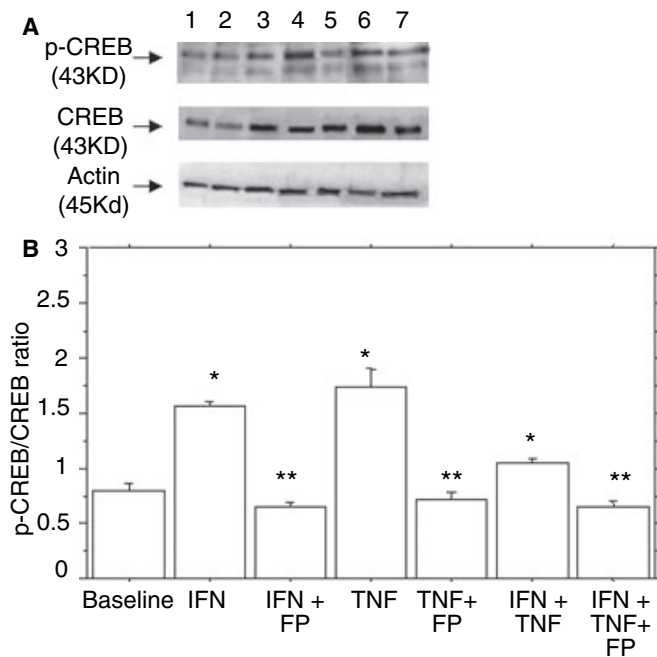


Figure 5. Effect of interferon- γ (IFN γ), tumor necrosis factor- α (TNF α) and fluticasone propionate on the expression of phospho-cyclic adenosine mono-phosphate response element-binding protein (p-CREB). Peripheral blood mononuclear cells (PBMC) isolated from normal donors ($n = 3$) were incubated with IFN γ (100 ng/ml) and with TNF α (10 ng/ml) for 30' alone and in combination and in the absence and in the presence of fluticasone propionate 10^{-7} M. Protein extracts were analyzed for p-CREB, CREB, and β -actin expression by western blot analysis in the same blot as above mentioned. (A) Representative western blot analysis. Lane 1, baseline; lane 2, IFN γ (100 ng/ml); lane 3, IFN γ (100 ng/ml) + FP 10^{-7} M; lane 4, TNF α (10 ng/ml); lane 5, TNF α (10 ng/ml) + FP 10^{-7} M; lane 6, IFN γ (100 ng/ml) + TNF α (10 ng/ml); lane 7, IFN γ (100 ng/ml) + TNF α (10 ng/ml) + FP 10^{-7} M. (B) Signals corresponding to p-CREB and CREB on the various western blots were semi-quantified by densitometric scanning, normalized for β -actin and results were expressed as p-CREB/CREB ratio. * $P < 0.05$ vs baseline. ** $P < 0.05$ vs the corresponding condition without fluticasone propionate (FP).

the expression of p-CREB was still present in SDA patients, characterized by a higher extent of airway inflammation and of epithelial cell abnormalities (13). It is known that these subjects, despite the treatment, have limited clinical and functional outcomes with a concurrent overproduction of inflammatory mediators (26). The findings that the SDA showed an increased expression of p-CREB both in the epithelium and in the submucosa as well as an increased CD45RB positive cell infiltration of the submucosa, demonstrate that the severity of asthma is related to the persistence of the pro-inflammatory phenotype despite the use of steroid therapy. Additional mechanisms may account for the permanence of an inflammatory status in bronchial airways of SDA. In this context, we previously demon-

strated that in bronchial epithelium (14) and in PBMC of SDA there was a higher expression of NF κ B which might enhance the inflammatory milieu and in turn the severity of the disease in this group of patients (27). The lack of endogenous anti-inflammatory mediator release, such as lipoxins, involved in the resolution of inflammation represents an alternative potential mechanism for this lack of control of inflammation (28).

Our findings demonstrating that p-CREB expression was increased in the submucosa and positively correlated with the number of CD45RB positive cells and the known concept that SDA patients are characterized by a persistent peripheral T-cell activation (29), prompted us to evaluate the expression of p-CREB and the p-CREB/CREB ratio in PBMC. We demonstrated in PBMC also that, the p-CREB expression and the p-CREB/CREB ratio were increased in UA and in SDA, further confirming the involvement of this molecule in the pathogenesis of this disease and supporting the concept of a 'systemic' involvement in bronchial asthma. This finding underlines that patients, receiving high dosage of inhaled glucocorticoids and being controlled by the treatment, may have some positive systemic effects from the treatment as demonstrated by the down-regulation of their p-CREB in PBMC. Although the activation of beta adrenergic receptors *in vitro* increases the binding of CREB to DNA, this phenomenon was not observed in an *in vivo* study on asthmatic patients (30). Accordingly, we established that ICS patients, regularly treated with β_2 agonists, exhibited a decreased p-CREB expression, suggesting that the effect of glucocorticoids on p-CREB is prevalent on the effect exerted by β_2 agonists. The finding that UA patients, not regularly treated with β_2 agonists, also exhibit p-CREB up-regulation, further supports the concept that the altered expression of p-CREB is linked to the inflammatory status, rather than to the activation of beta adrenergic receptors. In addition, it is possible to argue that the differences in the age of recruited patients probably did not influence the observed differences in p-CREB, as no studies specifically demonstrated an effect of age on the expression of p-CREB and as ICS and SDA or control and UA subjects in our study, not considerably different in age, differ in p-CREB expression.

For further insight into these mechanisms, we evaluated whether IFN γ and TNF α increased p-CREB expression and whether FP exerted a role in controlling this activation. In this set of experiments, we used normal PBMC to avoid any possible effects played by the above demonstrated status of activation of the CREB pathway in these cells from asthmatics and by the above demonstrated effects of the steroid therapy on this pathway. The findings that both IFN γ and TNF α , either alone or in combination, were able to significantly increase the expression of p-CREB, further underline the link between inflammation and this specific pathway. In this regard, it was previously demonstrated that exogenous IFN γ stimulates CREB phosphorylation and CREB-DNA

binding (21). This cytokine promotes immunity and modulates cell viability, in part by promoting alterations of cellular gene expression. The mechanisms by which TNF communicates with the nucleus and alters gene expression are not completely understood. In endothelial cells (human umbilical vein endothelial cells [HUVEC]), TNF α induces phosphorylation of the CREB transcription factor on serine 133 and increases CREB-DNA binding and transactivation (31). Dominant negative CREB, an antagonist antibody directed against the type 1 TNF receptor, or pharmacological inhibition of p38 mitogen activated protein kinases (MAPK) signaling blocked TNF-induced CREB activation as determined by phosphorylation and gene reporter assays (32). Here, we demonstrated the ability of FP to down-regulate, *in vitro*, the expression of p-CREB induced by both IFN γ and TNF α . Glucocorticoids have potent anti-inflammatory effects (33) and may inhibit pro-inflammatory gene products related to p-CREB activation in multiple ways. Activated GR monomers may bind either directly or indirectly with the transcription factor, preventing its ability to switch on inflammatory gene expression. The GR, as dimer, may bind to the GRE that overlaps the DNA-binding site for p-CREB or the start site of transcription, thus blocking gene expression or can impair the activity of MAPK, which, in turn, should reduce the phosphorylation of CREB, thus reducing its

DNA binding (34). In this regard, it was recently demonstrated that FP was able to reduce the expression of p-CREB and the DNA binding in fibroblasts stimulated *in vitro* with nicotine (35). Activated GRs may also down-regulate pro-inflammatory gene expression by binding to co-activators, including CBP, directly inhibiting their HAT activity or by down-regulating their expression (3, 6).

In conclusion, this study shows that in the bronchial epithelium, in the bronchial submucosa and in PBMC, the transcriptional factor p-CREB significantly increased in patients with bronchial asthma and its expression correlated with the inflammatory status. The anti-inflammatory effects of glucocorticoids are, at least partly, exerted by interfering with this pathway. Accordingly, p-CREB expression may be considered as the hallmark of inflammation in bronchial asthma.

Acknowledgments

This work was supported by the Italian National Research Council, by GlaxoSmithKline, by EU Framework programme for research, contract no. FOOD-CT-2004-506378, the GA²LEN project, Global Allergy and Asthma European Network and in part by INSERM-CNR contract. This work is dedicated to the memory of Antonio Maurizio Vignola.

References

- Imhof A, Wolffe AP. Transcription: gene control by targeted histone acetylation. *Curr Biol* 1998;**8**:422–424.
- Johnson CA, Turner BM. Histone deacetylases: complex transducers of nuclear signals. *Semin Cell Dev Biol* 1999;**10**:179–188.
- Barnes PJ, Adcock IM. Transcriptional factors and asthma. *Eur Resp J* 1998;**12**:221–234.
- Michael LF, Asahara H, Shulman AI, Kraus WL, Montminy M. The phosphorylation status of cyclic AMP-responsive activator is modulated via a chromatin-dependent mechanism. *Mol Cell Biol* 2000;**20**:1596–1603.
- Legube G, Trouche D. Regulating histone acetyltransferases and deacetylases. *EMBO Rep* 2003;**4**:944–947.
- Barnes PJ, Adcock IM. How do corticosteroids work in asthma? *Ann Intern Med* 2003;**139**:359–370.
- Struhl K. Histone acetylation and transcriptional regulatory mechanisms. *Genes Dev* 1998;**12**:599–606.
- Miller MK, Johnson C, Miller DP, Deniz Y, Bleecker ER, Wenzel SE, TENOR Study Group. Severity assessment in asthma: an evolving concept. *J Allergy Clin Immunol* 2005;**116**:990–995.
- Schwiebert LM, Stellato C, Schleimer RP. The epithelium as a target of glucocorticoid action in the treatment of asthma. *Am J Resp Crit Care Med* 1996;**154**:S16–S19.
- Redington AE, Wilson JW, Walls AF, Madden J, Djukanovic R, Holgate ST et al. Persistent airway T-lymphocyte activation in chronic corticosteroid-treated symptomatic asthma. *Ann Allergy Asthma Immunol* 2000;**85**:501–507.
- Bousquet J, Chanez P, Lacoste JY, Barneon G, Ghavanian N, Enander I et al. Eosinophilic inflammation in asthma. *N Engl J Med* 1990;**323**:1033–1039.
- Bateman ED, Frith LF, Braunstein GL. Achieving guideline-based asthma control: does the patients benefit. *Eur Respir J* 2002;**20**:588–595.
- Vignola AM, Chiappara G, Siena L, Bruno A, Gagliardo R, Merendino AM et al. Proliferation and activation of bronchial epithelial cells in corticosteroid-dependent asthma. *J Allergy Clin Immunol*, 2001;**108**:738–746.
- Vignola AM, Chanez P, Chiappara G, Siena L, Merendino A, Reina C et al. Evaluation of apoptosis of eosinophils, macrophages and T lymphocytes, in mucosal biopsy specimens of patients with asthma and chronic bronchitis. *J Allergy Clin Immunol* 1999;**103**:563–573.
- Chrivia JC, Kwok RP, Lamb N, Hagiwara M, Montminy MR, Goodman RH. Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature* 1993;**365**:855–859.
- Le Tulzo Y, Shenkar R, Kanedo D, Moine P, Fantuzzi G, Dinarello CA et al. Hemorrhage increases cytokine expression in lung mononuclear cells in mice: involvement of catecholamines in nuclear factor-kappaB regulation and cytokine expression. *J Clin Invest* 1997;**99**:1516–1524.

17. Thomas ML. The common antigen family. *Ann Rev Immunol* 1989;**7**: 339–369.
18. Bruno A, Chanez ., Chiappara G, Siena L, Giammanco S, Gjomarkaj M et al. Does leptin play a cytokine-like role within the airways of COPD patients? *Eur Resp J* 2005;**26**:398–405.
19. Gagliardo R, Chanez P, Vignola AM, Bousquet J, Vachier I, Godard P et al. Gluco-corticoid receptor alpha and beta in gluco-corticoid dependent asthma. *Am J Respir Crit Care Med* 2000;**163**: 7–13.
20. Rahman I, Gilmour PS, Jimenez LA, MacNee W. Oxidative stress and TNF-alpha induce histone acetylation and NF-kappaB/AP-1 activation in alveolar epithelial cells: potential mechanism in gene transcription in lung inflammation. *Mol Cell Biochem* 2002;**235**:239–248.
21. Liu L, Wang Y, Fan Y, Li CL, Chang Z. IFN gamma activates cAMP/PKA/ CREB signaling pathway in murine peritoneal macrophages. *J Interferon Citokine Res* 2004;**24**:334–342.
22. Barnes PJ, Adcock IM, Ito K. Histone acetylation and deacetylation: importance in inflammatory lung disease. *J Eur Resp* 2005;**25**:552–563.
23. Ito K, Caramori G, Lim S, Oates T, Chung KF, Barnes PJ et al. Expression and activity of histone deacetylases in human asthmatic airways. *Am J Respir Crit Care Med* 2002;**166**:392–396.
24. Merendino AM, Paul C, Vignola AM, Costa MA, Melis M, Chiappara G et al. Heat shock protein-27 protects human bronchial epithelial cells against oxidative stress-mediated apoptosis: possible implication in asthma. *Cell Stress Chaperones* 2002;**7**:269–280.
25. Barlow CA, Shuka A, Mossman BT, Lounsbury K. Oxidant-mediated cAMP response element binding protein activation: calcium regulation and role in apoptosis of lung epithelial cells. *Am J Resp Cell Mol Biol* 2006;**34**:7–14.
26. Adcock IM, Kazuhiro I. Steroid resistance in asthma: a major problem requiring novel solution or a non-issue? *Curr Opin Pharmacol* 2004;**3**:257–262.
27. Gagliardo R, Chanez P, Mathieu M, Bruno A, Costanzo G, Gougat C et al. Persistent activation of nuclear factor-kb signaling pathway in severe uncontrolled asthma. *Am J Respir Crit Care Med* 2003;**168**:1190–1198.
28. Vachier I, Bonnans C, Chavis C, Farce M, Godard P, Bousquet J et al. Severe asthma is associated with a loss of LX4, an endogenous anti-inflammatory compound. *J Allergy Clin Immunol* 2005;**115**:55–60.
29. Vrugth B, Djukanovic R, Bron A, Aalbers R. New insight into the pathogenesis of severe corticosteroid-dependent asthma. *Ann Allergy Asthma Immunol* 2002;**85**:501–507.
30. Hancox RJ, Stevens DA, Adcock IM, Barnes PJ, Taylor DR. Effects of inhaled β agonist and corticosteroid treatment on nuclear transcription factors in bronchial mucosa in asthma. *Thorax* 1999;**54**:488–492.
31. Gustin JA, Pincheira R, Mayo LD, Ozes ON, Kessler KM, Baerwald MR et al. Tumor necrosis factor activates CRE-binding protein through a p38 MAPK/ MSK1 signaling pathway in endothelial cells. *Am J Physiol Cell Physiol* 2004;**286**:C547–C555.
32. Lin MQ, Chang ZL. Immunomodulated signalling in macrophages regulation of the MAPK signaling pathways by PKA and PKC. *Sheng Wu Hua Yue Sheng Wu Wu Li Xue Bao (Shanghai)* 1999;**31**:701–706.
33. Walsh GM. Novel therapies for asthma – advances and problems. *Curr Pharm Des* 2005;**11**:3027–3038.
34. Adcock IM, Ito K. Gluco-corticoid pathways in chronic obstructive pulmonary disease therapy. *Proc Am Thorac Soc* 2005;**2**:313–319.
35. Tomic R, Lassiter CC, Ritzenthaler JD, Rivera HN, Roman J. Anti-tissue remodeling effects of corticosteroids: fluticasone propionate inhibits fibronectin expression in fibroblasts. *Chest* 2005;**127**: 257–265.