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Original Article

Nuclear Translocation of the Glucocorticoid Receptor in Fibroblasts of Asthmatic Patients with Nasal Polyposis Insensitive to Glucocorticoid Treatment

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ABSTRACT

Background: Nasal polyposis (NP) is treated with topical glucocorticoids (GC). Some patients require endoscopic sinonasal surgery because GC treatment is ineffective. To exert its function, the GC needs to bind with the GC receptor (GR) and the GC-GR complex moves to the cell nucleus. Our aim was to establish whether the poor response to GC is due to an alteration in the translocation of the GR to the nucleus.
Methods: Nasal fibroblast cell cultures were made from samples of seven healthy controls and 12 patients with NP and asthma. Fibroblasts were incubated with budesonide or dexamethasone (10^{-7} M) for different times (30 min to 4 h) and GR translocation was analyzed by immunocytochemistry.
Results: Both GCs induced GR translocation in every group, doubling its concentration in the cell nucleus (30 min) compared to baseline. There were no differences in GR translocation between controls and patients, nor differences related to the severity of asthma or intolerance to non-steroidal anti-inflammatory drugs (NSAIDs). Atopic subjects showed a decrease in GR translocation with budesonide (1 h, 3 h and 4 h, $p < 0.05$) and dexamethasone (30 min and 2 h, $P < .05$).
Conclusions: The insensitivity to GC treatment does not appear to be due to an alteration in GR translocation to the nucleus. Neither does asthma severity nor intolerance to NSAIDs appear to influence GR translocation. The association between atopy and the alteration in GR translocation merits further investigation.

Translocación nuclear del receptor de glucocorticoides en fibroblastos de pacientes asmáticos con poliposis nasal insensible al tratamiento glucocorticoideo

Palabras clave:
Asma grave
Poliposis nasal
Receptor de glucocorticoide

RESUMEN

Introducción: La poliposis nasal (PN) se trata con glucorticoides (GC) tópicos. En algunos pacientes el tratamiento es ineficaz requiriéndose cirugía endoscópica nasosinusal. Para ejercer su función, el GC precisa unirse al receptor de GC (RG) y este desplazarse al núcleo celular. Nuestro objetivo fue establecer si la pobre respuesta a los GC es debida a una alteración en la translocación del RG al núcleo.
Métodos: Se realizaron cultivos celulares de fibroblastos nasales de 7 controles sanos y 12 pacientes con PN y asma. Los fibroblastos se incubaron con budesonida o dexametasona (10^{-7} M) durante diferentes tiempos (30 min a 4 h) y la translocación del RG se analizó mediante inmunocitoquímica.
Resultados: Ambos GC indujeron translocación del RG en todos los grupos, doblando su concentración en el núcleo (30 min) respecto al basal. No encontramos diferencias en la translocación del RG entre controles y pacientes ni relación con la gravedad del asma o la intolerancia a los antiinflamatorios no esteroideos

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(AINE). En los sujetos atópicos se observó una disminución de la translocación con budesonida (1 h, 3 h y 4 h, p < 0,05) y dexametasona (30 min y 2 h, p < 0,05).
Conclusiones: La insensibilidad al tratamiento con GC no parece responder a alteraciones en la translocación del RG al núcleo. Tampoco la gravedad del asma ni la intolerancia a los AINE parecen influir en la translocación del RG. La asociación entre atopía y la alteración en la translocación del RG merece estudiarse más profundamente.

Introduction

Nasal polyposis (NP) associated with chronic rhinosinusitis is usually treated with topical glucocorticoids (GC) frequently associated with the continuous or intermittent use of systemic GC.[1,2] In a high percentage of patients, this treatment fails and nasal endoscopic surgery must be resorted to. In these cases, the nasal polyps represent an inflammatory disease that is either insensitive or resistant to GC. The resistance to GC can be observed in numerous inflammatory diseases, such as asthma, rheumatoid arthritis and ulcerative colitis.

Asthma is an inflammatory disease of the airways that is characterized by the presence of airflow obstruction and bronchial hyperresponse that varies in severity over the spectrum of the disease. Most asthma patients are easily diagnosed and respond to standard treatment with inhaled GC and short-acting β_2-agonists on demand for symptom control.[3]

There is, however, a subgroup of patients with severe asthma, which in some series reaches nearly 10% of asthmatics, that shows either poor or non-response to standard treatment and requires high dosages of GC to control their symptoms. These patients present greater morbidity and poorer quality of life than those whose pathology is correctly controlled with the treatment, meanwhile consuming 70% of the resources earmarked for this disease.[4,5]

The fact that severe asthma is defined and characterized by requiring high dosages of inhaled GC, occasionally associated with systemic GC, is thought to be related with the presence of insensitivity to these drugs. It can reach extreme values in so-called corticosteroid-resistant asthma.[6]

NP is frequently associated with severe asthma, especially in asthma with intolerance to non-steroidal anti-inflammatory drugs (NSAIDs).[7] Given the similar characteristics of the inflammation present in NP and in asthma, it can be inferred that the mechanisms responsible for the poor response to treatment with GC of the disease in the upper airways (NP and chronic rhinosinusitis) should be similar to those involved in insensitivity to these same drugs in serious asthma.

The mechanisms involved in the insensitivity to GC are partially understood. It is assumed that they are mostly acquired and induced by the inflammatory process, which would alter the normal function of the glucocorticoid receptor (GR) mediating the biological action of the GC.[6,8,9]

The GR is a transcription factor that belongs to the family of steroid receptors activated by hormones.[8-11] The GC molecule binds with the GR that is found in the cytoplasm associated with 2 subunits of heat-activated proteins, among others. The binding of GC with the GR causes the dissociation of these proteins and the activation of the GR, leading to the translocation of the GC-GR complex to the nucleus where it binds with specific DNA sequences, called glucocorticoid response elements (GRE), located in the promoter region of target genes. This union, together with certain co-activators, leads to the induction of the transcription of genes that code for anti-inflammatory proteins (trans-activation).[8,9,11-13]

The anti-inflammatory action of the GC can also take place through protein-protein interactions between an activated GR and nuclear transcription factors, such as AP-1 or nuclear factor NF-κB, repressing the inflammatory effects of these proteins (transrepression).[8,9,11-13]

As the nuclear translocation of the GR is an essential step for the GC to be able to exert their anti-inflammatory action, a possible mechanism involved in the insensitivity to treatment with GC in patients with NP could be the reduced nuclear translocation of the GR once activated by the hormone.[8,9]

The objective of our study was to research whether the poor response to GC of NP requiring surgical treatment is in part due to an alteration in GR translocation. To do so, we used nasal fibroblast cultures from healthy controls and from patients with NP associated with asthma of varying degrees of severity, with and without intolerance to NSAIDs. In *in vitro* studies on the response to GC, the drug usually used for stimulation is dexamethasone, although this is not the type of GC used for the treatment of NP and asthma in patients. For this reason, the nasal fibroblasts were also stimulated with budesonide, one of the GC used in daily clinical practice.

Methods

Subjects

Samples were obtained from the airways of 19 subjects, 7 healthy controls and 12 patients with NP and asthma. Asthma was classified by severity in accordance with the GINA criteria.[3] Nasal mucus samples were obtained from subjects who underwent corrective nasal surgery (nasal septoplasty or turbinectomy) and the NP of patients who had undergone nasal endoscopic surgery. All patients gave their written informed consent for the use of the tissue samples. The studied was approved by the Ethics Committee of the Clinic Hospital (Barcelona, Spain). The characteristics of the study population are shown in table 1.

Study of NSAID Intolerance and Atopy

All of the study subjects underwent clinical evaluation in order to identify those with a history compatible with intolerance to NSAIDs,

Table 1
Characteristics of the study population

	Control subjects	Mild asthma	Moderate-severe asthma
Patients (n)	7	3	9
Sex – females, n (%)	3 (42.8)	1 (33.3)	4 (44.4)
Age, years (mean ± SD)	34 ± 10	60 ± 11.7	49 ± 13.7
Nasal polyposis, n (%)	0 (0)	3 (100)	9 (100)
Intolerance to NSAID, n (%)	0 (0)	0 (0)	7 (77.7)
Atopy, n (%)	2 (28.5)	0 (0)	4 (44.4)
Smoking history, n (%)	0 (0)	0 (0)	2 (22.2)
Dosage of inhaled corticosteroids*	0	67 ± 115	764 ± 212
FEV1% FVC (mean ± SD)	80 ± 3.5	78 ± 2.8	69 ± 15.6
FEV1% (mean ± SD)	107 ± 7.5	102 ± 5	100 ± 13.2

*Budesonide or equivalent μg/d, mean ± SD.

and skin allergy prick-tests were used for common allergens (*Dermatophagoides pteronyssinus, Dermatophagoides farinae,* Gramineae, Cynodon, *Parietaria, Chenopodium,* Artemisia, Plantago, Olive, *Platanaceae,* Cypress, *Alternaria, Cladosporium, Aspergillus, Penicillium,* cat dander, dog dander, *Blatella orientalis, Latex*). In those cases where confirmation was required for the diagnosis of NSAID intolerance, the subjects underwent a nasal provocation test with lysine salicylate in accordance with a method developed by our group.[14]

Fibroblast Cultures

The nasal mucus and NP fragments were placed in 6-well culture plates with *Dulbecco's Modified Eagle's Medium* (DMEM) culture medium (Lonza, Belgium) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 0.002 mg/ml amphotericin B. They were kept in an incubator at 37 °C and 5% CO_2 for 3 weeks, changing the culture medium every 2 days. Using an optical microscope, fibroblast growth was confirmed and the first subculture was done by adding trypsin-EDTA. The subsequent subcultures were done when the cells reached a confluence of approximately 80% and the experiments were carried out with the 4th to the 8th subcultures. The presence of other cell types in the cultures was evaluated by vimentin immunocytochemistry (specific fibroblast marker) and cytokeratin (epithelial cell marker).

GR Immunocytochemistry

For the GR function study, the fibroblasts were cultivated in 8-well plates (BD Falcon) at a density of 10,000 cells/well in the presence of 500 μl of DMEM supplemented with 10% FBS treated with charcoal/dextran (FBScd, Gibco). This was done to eliminate the possible effect of GC and other endogenous hormones that the serum could contain on the translocation of the GR. Forty-eight hours afterwards, the cells were incubated with budesonide (BUD, Sigma) or dexamethasone (DEX, Fortecortín®, Merck), both at 10^{77} M, for different times (30 min, 1 h, 2 h, 3 h and 4 h).

After the incubation with GC, the cells were washed with cold PBS, they were set for 15 min in 4% paraformaldehyde in PBS and they were washed with PBS. They were permeated for 15ton X-100 diluted with 1% bovine serum albumin (BSA). After a 5-min wash with 1% PBS, we began the immunocytochemical localization of GR by means of incubation with a specific antibody for GR (sc-1003, Santa Cruz, USDA) diluted 1/100 in PBS at 1% BSA, incubating the whole night at 4 °C (125 μl per preparation). The following day, the samples were washed with (2 x 5 min), and we applied the fluorochrome-conjugated secondary antibody (Alexa 488, Molecular Probes, USA) diluted at 1/500 with PBS and 1% BSA (125 μl/well), 45 min at 37 °C (protected from light). Later, we carried out a 5-min wash with PBS and the preparations were mounted with a watery mounting medium (ProLong Gold, Molecular Probes, Invitrogen, USA), left to solidify at least 3 h at room temperature protected from light. Fluorescence microscopy (Leica Microsystems, Germany) was used to observe GR in the samples. For each of the experimental conditions, the translocation of GR was quantified in 30 to 100 cells by analyzing the mean fluorescence intensity (MFI) emitted by the GR after processing the images with *Image J* software. The results were expressed as the proportion of MFI of the cell nucleus in each of the analysis conditions compared with baseline fluorescence (prior to incubation with the hormone).

Statistical Analysis

After studying the distribution characteristics of the variables, non-parametric tests were used (Kruskal-Wallis, U Mann-Whitney). A p value < 0.05 was considerer statistically significant.

Results

In the absence of GC, we confirmed by means of immunocytochemistry that GR presented a uniform distribution in the cytoplasm and nucleus of the fibroblasts of the upper airways of all the samples studied (fig. 1A).

In the presence of GC, the fibroblasts were incubated with each GC (BUD or DEX, 10^{77}) for different times (30 min, 1 h, 2 h, 3 h and 4 h) in order to be able to establish the chronology of the translocation of GR to the nucleus, confirming early response as it was detected 30 min after incubation with GC in all the samples analyzed (fig. 1B).

The presence of GR translocation to the nucleus was observed in the samples of the 19 subjects analyzed after stimulation with BUD as well as with DEX. The depletion of GR of the cytoplasm was associated with the corresponding increase in accumulation of GR in the nucleus (fig. 1).

Afterwards, we analyzed the translocation of GR in different patient subgroups. To begin with, we analyzed the translocation of nasal fibroblast GR depending on the severity of the asthma according to criteria established by GINA,[3] as observed in figure 2. The translocation of the GR to the nucleus is already detected 30 min after the administration of BUD (fig. 2A) or DEX (fig. 2B), doubling the mean fluorescence intensity (MFI) of the nucleus compared with the baseline fluorescence. The GR remains in the nucleus until 4 h after the administration of the drug in all the groups ($p < 0.05$ compared with baseline).

No differences were observed in GR translocation induced by either BUD (fig. 2A) or DEX (fig. 2B), according to asthma severity. In the group of patients with moderate-severe asthma, we observed a tendency towards less GR translocation induced by BUD compared with the mild asthma patient group, although without reaching statistical significance (fig. 2A).

The following is an analysis of the translocation of GR depending on the presence or absence of intolerance to NSAIDs (fig. 3). We observed no significant differences in the translocation kinetics of GR induced by BUD (fig. 3A) or DEX (fig. 3B) among patients who were either tolerant or intolerant of NSAIDs.

Last of all, we analyzed the translocation of GR depending on the presence of atopy, as observed in figure 4. GR translocation, induced by BUD (fig. 4A) as well as by DEX (fig. 4B), was less in the atopic subjects when compared with the non-atopic ones.

Discussion

The aim of our study was to further investigate the molecular mechanism implicated in the lack of response to GC treatment in NP, and specifically whether in these patients there is an alteration in GR activation. For this reason, we analyzed the behavior of the GR in the nasal mucus of healthy subjects an in the NP of patients who had undergone surgery due to failure of GC treatment. We interpreted in these cases that the need for surgery revealed the presence of insensitivity or resistance to GC. Given that NP is frequently associated with severe asthma and with NSAID-intolerant asthma, we also analyzed the activation of GR depending on asthma severity and presence of NSAID intolerance.

The binding of the GC to the GR in the cytoplasm induces the formation of a GC-GR dimer that translocates to the nucleus and acts as a transcription factor, activating or inhibiting the transcription of the target genes. The translocation of GR to the nucleus constitutes an essential step in order for the GC to exert their anti-inflammatory effects.[8,9,11,12] In our study, we analyzed the translocation of GR in fibroblast cultures obtained from healthy nasal mucus and nasal polyps of asthma patients. Obtaining the samples during nasal surgery with the patient under general anesthesia has allowed us to minimize the risk involved in performing bronchial biopsies or

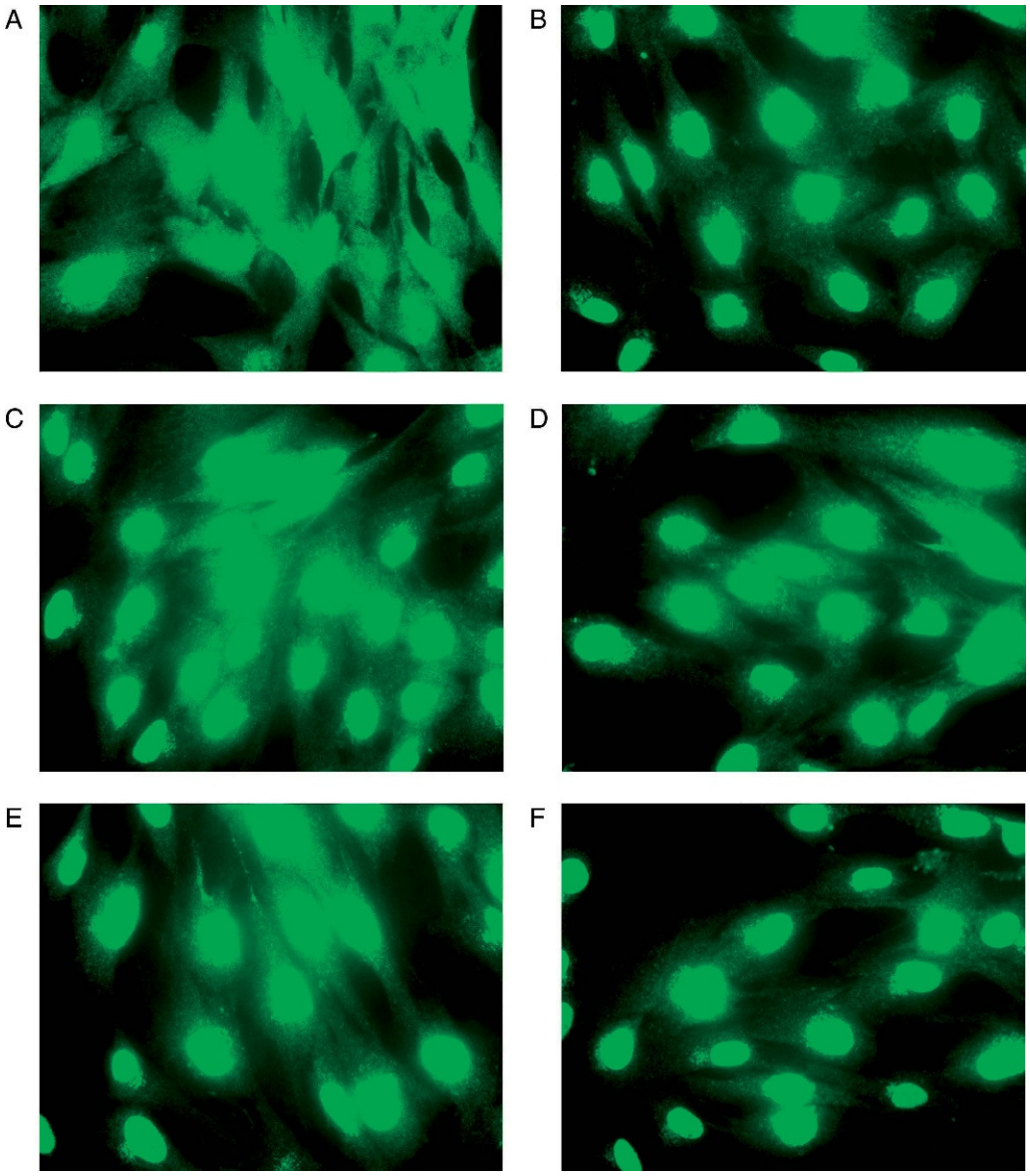

Figure 1. Image showing the translocation of GR in nasal mucus fibroblasts obtained using fluorescence microscopy. Prior to the incubation of the fibroblasts with GC (A), the GR is located in the cytoplasm as well as the nucleus. After incubation with GC (BUD) for 30 min (B), 1 h (C), 2 h (D), 3 h (E) and 4 h (F), immunofluorescence corresponding to GR is detected in the nuclei of the fibroblasts.

bronchoalveolar lavage by means of bronchoscopy in asthma patients, especially in the subgroup of severe patients. The concept of "one airway, one disease" claims that the mechanisms involved in the inflammatory disease of the upper airways (rhinitis, sinusitis and nasal polyposis) are similar to those of the lower airways (asthma). Likewise, the treatments applied in nasal and bronchial diseases are also the same type; therefore, therapeutic failure can arise in both processes and it is logical to suppose that the mechanisms responsible are similar in both parts of the airways.[15] According to this premise, the study of the cells obtained from the upper airway of our asthma patients is comparable to the affectation that these same patients present in their lower airway.[16]

First of all, we confirmed by means of immunocytochemistry that, in the absence of GC, the GR presented a uniform distribution in the cytoplasm and was also observed in the nucleus of the fibroblasts of the upper airway of all the samples studied. In keeping with these findings, Usmani et al.[17] gave evidence of the presence of GR in the cell nucleus (30%) of epithelial cells and macrophages of patients with mild asthma prior to stimulation with fluticasone *in vivo*, attributing this to a reflex of the response to the endogenous levels of circulating cortisol. To minimize the effects of the possible endogenous cortisol that the culture medium may contain, in our series all translocation studies were carried out with a culture medium in which FBS was treated with charcoal/dextran. In spite of this, we also found evidence of the presence of GR in the nucleus of all the samples. This effect could be attributed to different causes: one possibility would be that the wash time with FBScd was insufficient, which would justify the presence of GC in the cell nucleus prior to incubation with BUD or DEX. It could also be explained by the type of antibody used for GR detection, as we used an antibody that, in addition to detecting functional GR (GRα), also detects the β isoform of GR. Given that GRβ seems to be located only in the nucleus, the GR located in the nucleus prior to the addition of GC that we observed in our study could be due to the detection of GRβ.[8] Nevertheless, several researchers have demonstrated that the levels of expression of GRβ are negligible compared with those of

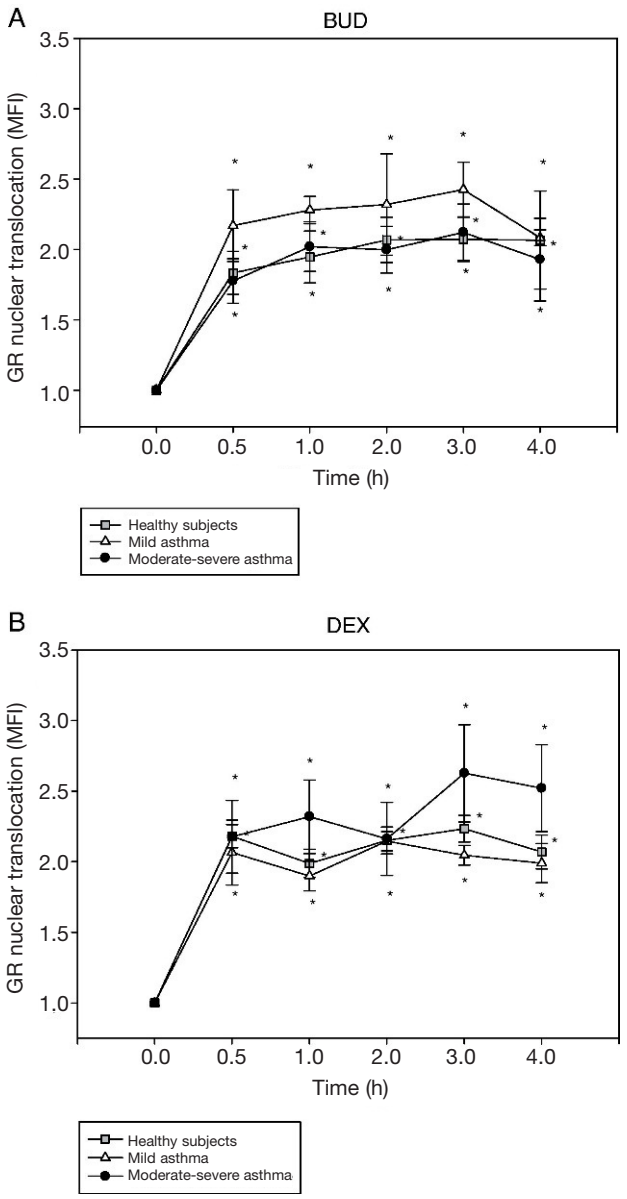

Figure 2. Effect of the incubation with BUD (A) and DEX (B) on the translocation of GR in fibroblasts of healthy subjects, patients with mild asthma and patients with severe asthma. No statistically significant differences were observed in GR translocation induced by either BUD or DEX among the different patient groups (*p < 0.05 all the groups compared at 0 h, Wilcoxon).

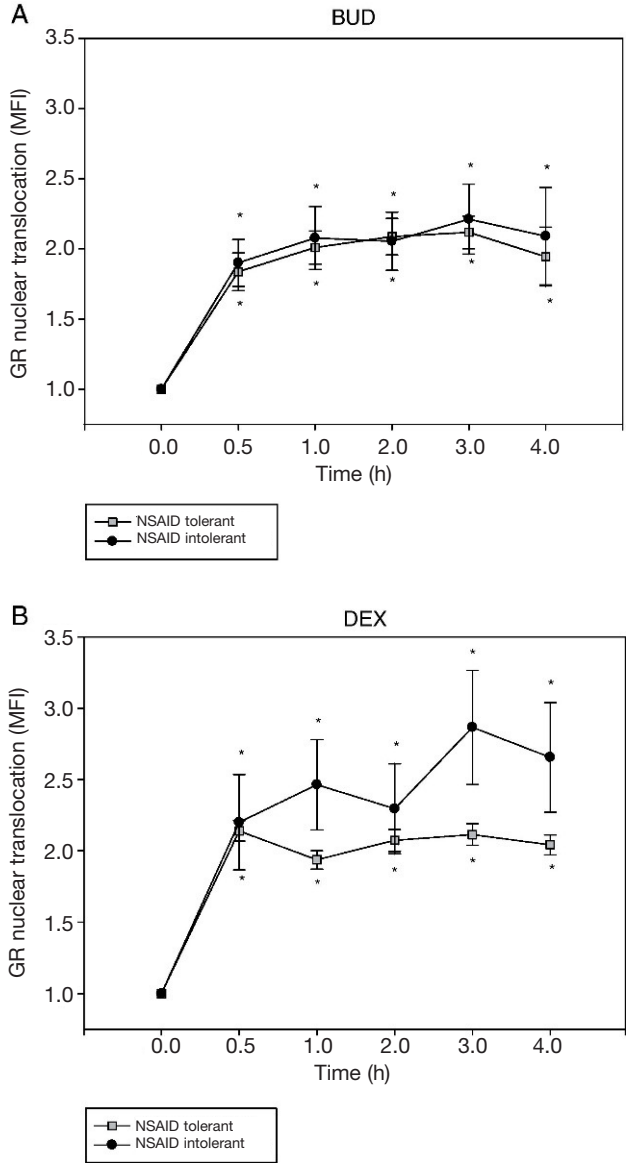

Figure 3. The effect of incubation with BUD (A) and DEX (B) in the GR translocation in the fibroblasts of subjects tolerant and intolerant to NSAIDs. No statistically significant differences were observed in the translocation of GR induced by either BUD or DEX between NSAID-tolerant or intolerant subjects (*p < 0.05 all the groups compared at 0 h, Wilcoxon).

GRα.[8,9,11,13] In accordance with our findings in the absence of DEX, Goleva et al.[18] also observed the presence of GR in the cell nucleus of macrophages in the bronchoalveolar lavage of patients with asthma, whether sensitive or insensitive to GC.

Just like the results obtained by other researchers in epithelial cells and macrophages obtained from the sputum of healthy subjects and patients with mild asthma,[19] we also observed a rapid translocation of GR (30 min) in the fibroblasts of our patients. Likewise, we have also shown that the prolonged incubation with GC up to a maximum of 4 h did not cause an increase in GR translocation to the nucleus, suggesting that at brief exposition to the hormone is enough to activate GR.

We did not find differences in the translocation of GR between the fibroblasts of healthy subjects and those with NP. Nor did we observe differences between the fibroblasts coming from patients with mild and severe asthma. Likewise, we did not find that the presence of NSAID intolerance had any influence in the translocation of GR. Given that there are several mechanisms implicated in the lack of response to the treatment with GC, we can conclude that the mechanism responsible for the poor response of the NP to the GC is not explained by alterations in the translocation of GR to the nucleus.

One of the possible biases of the study that would justify the absence of differences in the translocation between healthy subjects and patients with asthma of different severities could be related with the characteristics of our study population. Our group of asthma patients presented correct symptom control despite their varying degrees of asthma severity, and in the case of patients with moderate-severe asthma, the patients did not have great functional affectation nor did they require high dosages of treatment to maintain stability. Perhaps there is an alteration of the GR response in those patients with poor asthma control despite treatment or with more severe

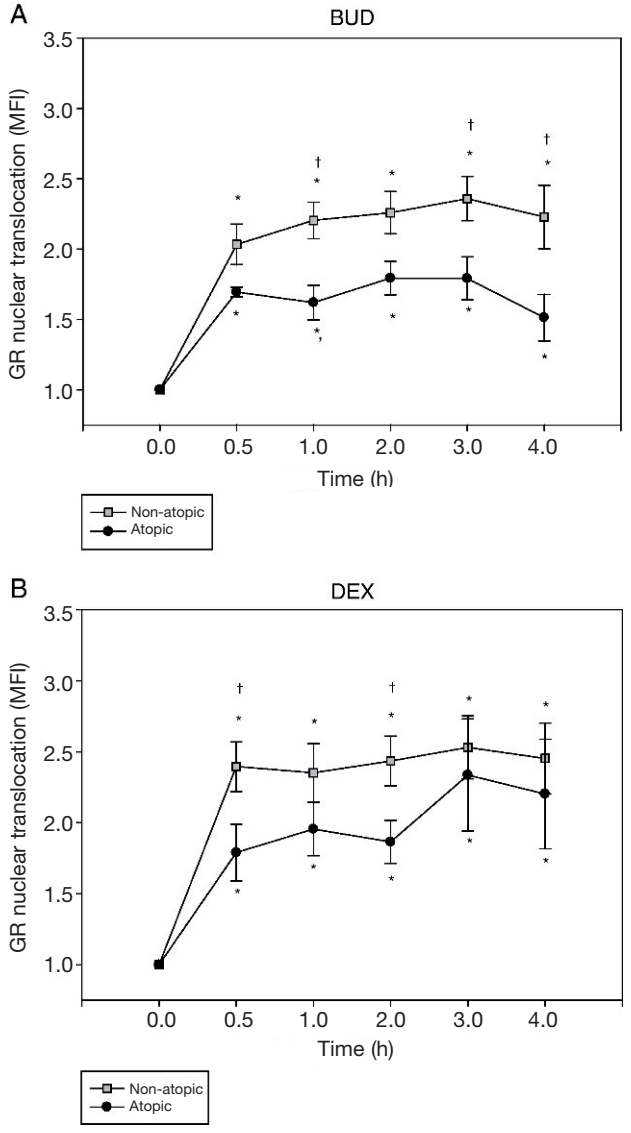

Figure 4. The effect of incubation with BUD (A) and DEX (B) in the translocation of GR in the fibroblasts of atopic and non-atopic subjects. Less GR translocation is observed, induced by BUD (A) as well as by DEX (B), in the atopic subjects compared with non-atopic subjects in each of the different incubation times studied, reaching statistical significance at 1 h, 3 h and 4 h with BUD and 30 min and 2 h with DEX (†$p < 0.05$ compared with non-atopics, Mann-Whitney) (*$p < 0.05$ compared at 0 h, Wilcoxon).

asthma. A potential limitation of our study would be the sample size, which could partially explain the lack of differences between the groups.

Our results contrast with those obtained by Goleva et al.[18] In macrophages obtained by bronchoalveolar lavage, these authors[18] reported less translocation of GR induced by DEX (10^{-6} M, 3 h) in the cells of patients with asthma insensitive to GC, compared with that of patients sensitive to GC. Not having found similar differences in our patients could be related with the cell type that was studied. Our study deals with structural cells, fibroblasts, which participate in the remodeling of the airways. In contrast, Goleva et al.[18] studied alveolar macrophages, which are cells involved in the activation and maintenance of the inflammation of the airways that in asthma show a greater capacity for presenting antigens.[20,21]

The prevalence of allergies in the adult asthmatic population ranges between 30 and 40%.[22] On a practical level, it is considered that atopic patients are those that show sensitization to an allergen during skin prick tests or radioallergosorbent tests (RAST). The exposure to allergens has been widely reported as an important risk factor for the development of asthma and severe asthma in particular.[23,24] For this reason, we contemplated sensitization using prick tests in our study population and we found evidence that the presence of atopy was accompanied by a statistically significant decrease in GR translocation to the nucleus, after incubation with BUD as well as with DEX. Our findings concur with a previous study that examined the effects of allergens on the affinity of the binding of the GC to the GR in mononuclear cells of the peripheral blood.[25] These authors demonstrated that the *in vivo* as well as *in vitro* exposure to an allergen reduced the binding affinity of GC to GR in allergic asthmatics, with the resulting functional alteration in cell response to GC. The mechanism by which this alteration in the affinity of GR would be produced is unknown. For some authors,[25] in the case of T cells this alteration would seem to be mediated by IL-2 and IL-4. Thus, the exposure to the allergen would cause an activation of the immune system and an increase in the expression of these cytokines, which would in turn increase the expression of the isoform β of the GR in the T cells, which has been seen to be involved in a lesser response to the endogenous and exogenous GC.[8,9] In previous studies, however, our group did not find differences in GR-β expression in asthma patients, which would not support the hypothesis of its implication in the lack of response to GC.[13,26]

In short, in our study we did not find differences in GR translocation between the fibroblasts of healthy nasal mucus and fibroblasts from NP. Nor did we observe that the severity of the asthma or the presence of intolerance to NSAIDs had any influence either. We can therefore conclude that the lack of response to treatment with GC cannot be attributed to an alteration in the translocation of GR. In contrast, we did demonstrate less GR translocation in atopic patients. The origin of this association is unknown and will require further studies.

Conflict of Interest

The authors declare having no conflict of interest.

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