

Original article

Glucocorticosteroids rapidly inhibit allergen-induced expression of E-selectin *in vitro* in a mucosal model of allergic rhinitis

Background: Transendothelial migration of cells to sites of inflammation is a hallmark of the allergic reaction. The adhesion cascade involves the initial expression of the adhesion molecule E-selectin on endothelial cells. The aim of the study was to determine the efficacy of a 30-min preincubation of the glucocorticosteroids (GCS) fluticasone, prednisolone, and flucortin butyl on allergen- and interleukin (IL)-1 β -induced E-selectin expression in allergic rhinitis.

Methods: Freshly taken nasal inferior turbinate mucosa of 19 subjects with allergic rhinitis was cut into small cubes and preincubated for 30 min with prednisolone ($n=6$), fluticasone ($n=5$), and flucortin butyl ($n=3$) in different concentrations, followed by allergen exposure at a concentration of 1000 BU/ml for 1 and 2 h. Additionally, fluticasone-preincubated tissues were exposed to recombinant human rhIL-1 β ($n=5$) at a concentration of 2 pg/ml. The expression of E-selectin was assessed by immunohistochemistry (APAAP technique) and computerized image evaluation.

Results: In this model, E-selectin expression was significantly upregulated by allergen and rhIL-1 β within 1 and 2 h. After 30-min preincubation with prednisolone and flucortin butyl at drug concentrations of 10^{-8} mol/l, we found a significant ($\geq 50\%$) reduction of the E-selectin expression after 1 and 2 h. Allergen-induced E-selectin expression was nearly abolished at concentrations of 10^{-5} (prednisolone) and 10^{-4} mol/l (flucortin butyl). Fluticasone significantly inhibited E-selectin expression by $\geq 50\%$ at concentrations of 10^{-14} and 10^{-12} mol/l after 1 and 2 h, and abolished E-selectin induction at concentrations of 10^{-12} and 10^{-10} mol/l, respectively. Exposure of mucosal cubes to rhIL-1 β ($n=5$) also induced rapid upregulation of E-selectin expression, an effect which could be only partially suppressed by fluticasone preincubation at concentrations of 10^{-10} mol/l.

Conclusions: Allergen-induced E-selectin expression is significantly and rapidly inhibited by GCS preincubation, fluticasone being more potent than prednisolone and flucortin butyl. We suggest that this rapid effect is mainly indirect, possibly by inhibition of allergen-induced cytokine release.

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Allergic rhinitis has been characterized as an inflammatory reaction involving the synthesis and release of cytokines, the activation of the adhesion cascade, and the transendothelial migration of inflammatory cells such as eosinophils, basophils, and mononuclear cells. Proinflammatory cytokines, such as IL-1 β and TNF- α , are released rapidly after allergen exposure and play a prominent role in the ongoing inflammatory reaction by activating endothelial cells and T cells (1, 2). Among other activities, they induce the expression of adhesion molecules such as E-selectin, ICAM-1, and VCAM-1 on endothelial cells, an effect which may lead to an increased transendothelial migration of eosinophils,

neutrophils, mononuclear cells, and basophils from the blood circulation. Within the adhesion cascade, selectins mediate the initial rolling of leukocytes along the vascular wall, exposing the cells to further signals by chemokines and to another group of adhesion molecules, the immunoglobulin supergene family (2, 3). A significant upregulation of adhesion-molecule expression has been demonstrated within the nasal mucosa of seasonal and perennial allergic rhinitis patients (1, 4).

Topical glucocorticosteroids (GCS), as the most potent anti-inflammatory drugs available, are frequently used in the treatment of allergic rhinitis. They have been demonstrated to inhibit strongly

cytokine synthesis by and release from a range of cells, and to downregulate inflammatory processes effectively by direct and indirect effects on cell activation and migration (5, 6). Thus, several studies have convincingly demonstrated the inhibition of eosinophil migration and eosinophilic mediator release in allergic rhinitis (7–9). As GCS mainly act by inhibition of protein synthesis, affording the uptake of the drug into the cell nucleus and the inhibition of gene transcripts, it takes several hours for their action to be effective. However, immediate actions have also been described (e.g., blocked ACTH secretion) and related to non-receptor-mediated effects (5, 6, 10).

The aim of our study was to determine the potentially rapid effects of GCS such as fluticasone, flucortin butyl, and prednisolone on E-selectin expression, representing the initial step in the adhesion cascade, in allergic nasal mucosa. We used an *ex vivo* culture model, stimulating inferior turbinate mucosa of seasonal allergic rhinitis patients with the grass-pollen allergen to which these subjects were sensitized. By comparing the effects of GCS on E-selectin expression due to allergen or rhIL-1 β exposure, we investigated whether GCS have a direct or indirect effect on E-selectin expression.

Material and methods

Subjects

Nasal mucosal biopsy specimens from nasal inferior turbinates of 19 grass-pollen-allergic patients (18–60 years of age, mean 35 years) were obtained outside the pollen season during routine surgical procedures for medical reasons (septal deviation and conchal hypertrophy). Allergy to grass pollen was confirmed by skin prick test and patient history. None of the subjects had received systemic or topical GCS, antihistamines, or immunotherapy within 4 weeks prior to the experiments. Informed consent was obtained from all the subjects, and the study was approved by the ethics committee of the University of Düsseldorf (Germany).

Tissue-exposure experiments

Biopsies, cut into small cubes of about 2 mm³, were transferred in RPMI medium enriched with 10⁻¹² mol/l prednisolone to inhibit baseline E-selectin expression due to the surgical trauma, and were kept at 37°C. Prednisolone (Merck, Darmstadt, Germany), fluticasone propionate (Glaxo Wellcome, London, UK), and flucortin butyl (Scherax, Hamburg, Germany) were then added for 30 min, as detailed below, before stimulating the tissues by exposure to allergen (1000 biologic units/ml lyophilized grass-pollen mixture, Allergopharma J. Ganzer, Hamburg, Germany) or recombinant human (rh)IL-1 β (2 pg/ml, R&D Systems, Inc., Minneapolis, MN, USA). Pilot experiments were

performed before this study to find the optimal prednisolone concentration to suppress trauma-induced E-selectin expression (maximum 20%) and to establish concentrations of allergen and rhIL-1 β to induce a minimum of 50% E-selectin expression. Each tissue stimulation series was paralleled by controls (baseline expression, no stimulation, and allergen stimulation without GCS).

Immunohistochemistry

At 1 and 2 h after allergen or rhIL-1 β exposure, the tissue cubes were fixed in Formaline and later cut into 5- μ m slides. Specimens were stained in serial sections by the APAAP technique. E-selectin was visualized by a monoclonal antibody CD62E (clone BBIG-E4, R&D Systems Inc., Minneapolis, MN, USA) and compared to a stain of total endothelial cells by an antibody to von Willebrand factor (Dianova, Hamburg, Germany).

Data analysis

The mean percentage of E-selectin-positive endothelial cells within three subepithelial areas was calculated on the basis of the total number of endothelial cells in adjunct serial sections by an image analysis system (SIS, Münster, Germany).

Statistics

The results given in Figs. 1–4 were determined as mean values and SEM of all experiments. Significance was determined by a one-way ANOVA analysis (one-sided). Significance was accepted for $P \leq 0.05$.

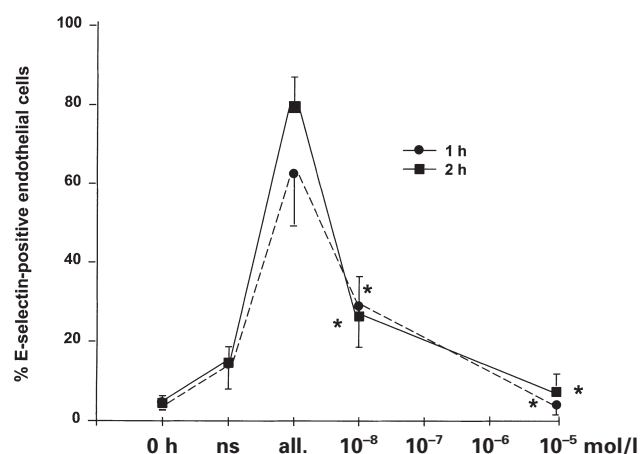


Figure 1. Action of prednisolone on allergen-induced E-selectin expression ($n=6$). ns: nonstimulated control; all.: stimulation with allergen. * $P > 0.05$ vs allergen stimulation.

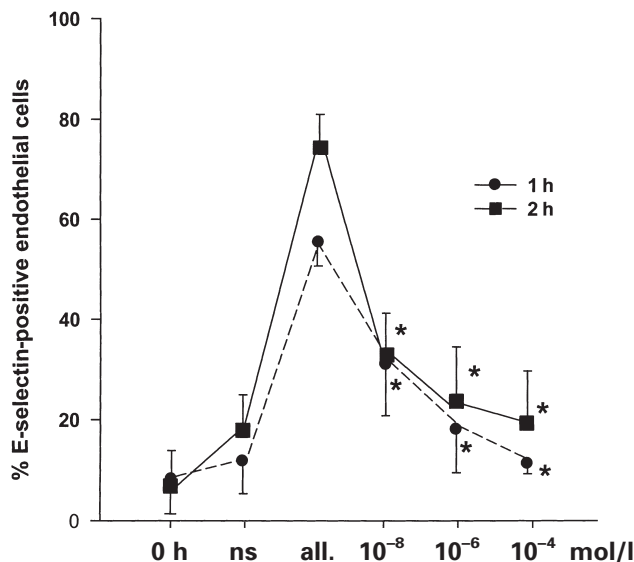


Figure 2. Action of flucortin butyl on allergen-induced E-selectin expression ($n=3$). ns: nonstimulated control; all.: stimulation with allergen. * $P>0.05$ vs allergen stimulation.

Results

Allergen and rhIL-1 β stimulation

Whereas baseline expression of endothelial cell E-selectin was regularly below 10% and controls without allergen stimulation were below 25% of expression of von Willebrand factor at 1 and 2 h, allergen exposure caused an induction of E-selectin mean expression of 51–84% and 61–96%, respectively. rhIL-1 β stimulation led to a mean increase of E-selectin expression to 69% and 72% after 1 and 2 h. Allergen- and rhIL-1 β -induced stimulation data served as baseline for all calculations on suppressive effects of GCS.

Prednisolone

After preincubation with prednisolone, we found a significant reduction of the mean E-selectin expression from 79% to 8% ($n=6$) after 2 h and from 62% to 14% after 1 h at a concentration of 10^{-5} mol/l (Fig. 1). A significant 50% suppression of E-selectin induction after 1 and 2 h was seen at a concentration of 10^{-8} mol/l.

Flucortin butyl

Flucortin butyl suppressed E-selectin expression significantly after 2 h of incubation from 75% to 19%, and after 1 h of incubation from 54% to 11% at a concentration of 10^{-4} mol/l. A significant 50% reduction of E-selectin induction was achieved at a concentration of 10^{-8} mol/l (Fig. 2).

Fluticasone

In contrast to flucortin butyl and prednisolone, concentrations as low as 10^{-10} mol/l fluticasone

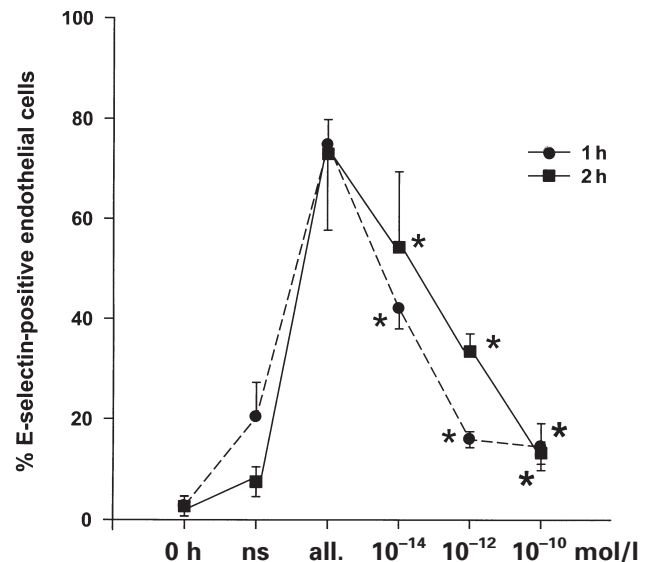


Figure 3. Action of fluticasone on allergen-induced E-selectin expression ($n=5$). ns: nonstimulated control; all.: stimulation with allergen. * $P>0.05$ vs allergen stimulation.

($n=5$) suppressed E-selectin expression significantly from 76% and 74%, respectively, to 15% after 1 and 2 h (Fig. 3). The inhibitory concentration for 50% reduction of E-selectin induction ranged from 10^{-14} to 10^{-12} mol/l for 1 and 2 h. In contrast, when the nasal mucosa was stimulated with rhIL-1 β ($n=5$), fluticasone at a concentration of 10^{-10} mol/l induced only a 28% and 36% suppression of E-selectin expression after 1 and 2 h, respectively (Fig. 4).

Discussion

In recent years, allergic rhinitis has been clearly recognized as an inflammatory reaction involving the release of cytokines and chemokines, the expression of adhesion molecules, and the recruitment and activation of inflammatory cells. A key feature in this reaction is the activation of the adhesion cascade, a complex, stepwise process to recruit leukocytes from the peripheral blood into the target tissue by expressing a variety of specific adhesion molecules (2, 3). Selectins, especially E-selectin, are upregulated after activation of endothelial cells by cytokines such as IL-1, TNF- α , and other factors, and mediate the first step in the adhesion cascade. Due to the expression of these adhesion molecules, the endothelium functionally displays increased adhesive properties for circulating leukocytes, which are loosely bound to the vascular wall to await further signals (3). In allergic inflammation, it has been revealed that cytokines, namely, TNF- α and IL-1 β , classified as nonspecific endothelial activators, are released during experimental antigen challenge and natural antigen exposure (1, 11, 12). The rapid release of IL-1 β and TNF- α into nasal secretions within 1–2 h

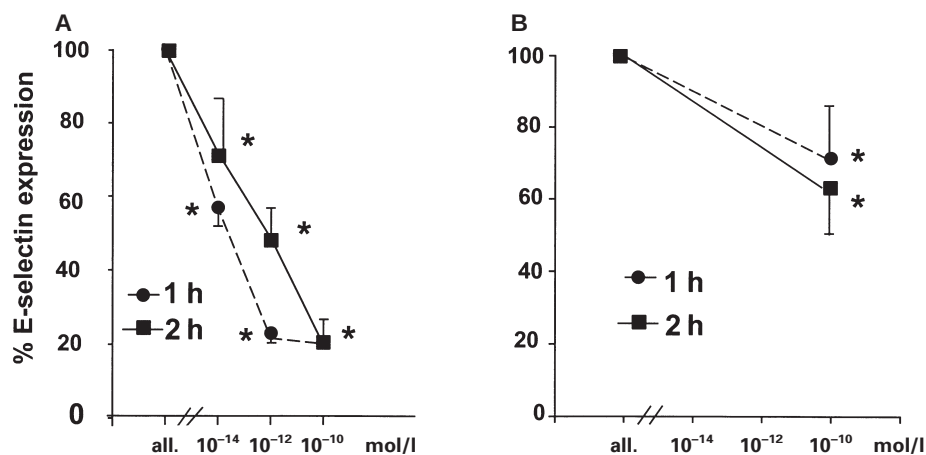


Figure 4. Action of fluticasone on E-selectin expression due to stimulation with allergen (A) ($n=5$) or rhIL-1 β (B) ($n=5$). * $P>0.05$ vs allergen or rhIL-1 stimulation. all.: stimulation with allergen.

after allergen challenge suggests that these proinflammatory cytokines are secreted from cellular stores rather than being newly synthesized (11–15).

There is strong evidence that the induction of adhesion molecules is also a hallmark of naturally occurring allergic rhinitis. In perennial allergic rhinitis patients, an upregulation of ICAM-1 and VCAM-1 has been shown as compared to controls, and in seasonal allergic rhinitis, we found a significant upregulation of E-selectin and ICAM-1 on endothelial cells as compared to controls (1, 4, 16).

Here, we present an *ex vivo* model using inferior turbinate mucosa, in which we were able to demonstrate an induction of E-selectin expression due to exposure to grass-pollen allergen in grass-pollen-sensitized subjects, whereas exposure to nonrelevant allergens did not induce E-selectin expression (unpublished data).

In our model, E-selectin expression was also effectively inducible by rhIL-1 β stimulation within the same time frame. As endothelial cells do not respond directly to allergen, these findings suggest that allergen induces the release of cytokines such as IL-1 β and TNF- α and indirectly induces E-selectin expression on endothelial cells. The source of the cytokines involved was not clarified, but apart from mast cells, macrophages may also be good candidates, as they bear IgE receptors on their surface and respond to allergen stimulation with the release of proinflammatory cytokines (17, 18).

In several pilot studies performed with the *ex vivo* model, we found a significant reduction of E-selectin expression by GCS when given at various time points before allergen exposure. For the final experiments, we were able to select a preincubation time of only 30 min before allergen or rhIL-1 β challenge of the tissue. Prednisolone, flucocortin butyl, and fluticasone elicited a significant dose-dependent inhibition of E-selectin expression after 1 and 2 h, with relevant differences in concentrations needed for the three GCS. A

statistically significant 50% suppression of E-selectin induction was achieved at concentrations of 10^{-8} mol/l for prednisolone and flucocortin butyl, whereas the respective concentration for fluticasone was only 10^{-14} – 10^{-12} mol/l (56% ELAM expression at 10^{-14} mol/l after 1-h exposure to stimulus, and 48% ELAM expression at 10^{-12} mol/l after 2 h, respectively). Thus, it was possible to show differences in the efficacy of the different GCS in terms of the concentration necessary to reduce markedly allergen-induced E-selectin expression.

When we used stimulation with rhIL-1 β , we could demonstrate an induction of E-selectin expression in the mucosal model comparable to that of allergen stimulation. However, the inhibitory activity of fluticasone under these conditions was limited to about 30–40%, when compared to the suppression of allergen-induced E-selectin expression. These findings suggest that, under the conditions of this experiment, fluticasone acts indirectly on E-selectin expression by reducing the release of stimuli from source cells. In line with our findings, Johnson could demonstrate that fluticasone reduces TNF- α -induced E-selectin expression on human endothelial cells in culture (15% inhibition at a concentration of 10^{-10} mol/l fluticasone) (19). Other *in vitro* studies revealed that GCS do not inhibit the expression of E-selectin induced by IL-1, nor do they inhibit the acquisition of adhesion properties for eosinophils, basophils, or neutrophils in a model in which the cytokines were supplied exogenously (20, 21).

As GCS have been shown to be potent inhibitors of production and release of a range of cytokines including IL-1 β and TNF- α (22–25), they are probably responsible for indirect inhibition of E-selectin expression. Recently, a clinical study by Weido et al. (26) showed that intranasal fluticasone inhibited recovery of chemokines and cytokines such as IL-1 β , IL-6, and IL-8 in nasal secretions in allergen-induced rhinitis. Furthermore, fluticasone inhibited cytokine production

of IL-3 and IL-5 and nasal symptoms in ragweed-allergic rhinitic patients (7).

However, steroids may also have a direct inhibitory effect on the expression of adhesion molecules, as was shown for ICAM-1 and E-selectin at the level of gene transcription (8). Fluticasone inhibited the expression of adhesion molecules such as E-selectin on human endothelial cells in culture, if stimulated by TNF- α (19). This would suggest at least a partially direct effect on E-selectin expression under certain treatment conditions. However, our *ex vivo* model pointed to indirect effects of fluticasone as a major contribution to the overall efficacy in a short-term setting.

GCS produce their effects on responsive cells by binding to cytoplasmic glucocorticoid receptors, and then being transferred into the nucleus to regulate transcription of certain target genes. This may happen either directly by binding to DNA at consensus sites termed glucocorticoid response elements (GRE) or indirectly by binding to AP-1 (transcription factor activator protein-1) or NF- κ B (nuclear factor-kappa B), preventing transcription by inactivating transcription factors. Both modes of action have been demonstrated to be relevant to the suppression of cytokine production (8, 27, 28). Furthermore, non-receptor-mediated effects have been described in models, such as the cytotoxic response in thymocytes and the reduction of vascular exudation in rodents, in which the steroid needs to be present in tissue for only 5–10 min (10). Such membrane-stabilizing effects may contribute to the effects seen in our model.

GCS may show various activities according to their pharmacodynamic and pharmacokinetic properties.

Steroid action is dependent upon the lipophilicity, tissue uptake and distribution, receptor affinity, and kinetics. Fluticasone propionate is more lipophilic than the two other GCS tested and shows a higher drug uptake and retention in tissue (29). In terms of affinity for the human glucocorticoid receptor, fluticasone has a higher affinity than the others and shows a low rate of dissociation from the receptor, resulting in a half-life of the receptor complex in excess of 10 h. These differences in pharmacokinetic properties may account for the difference in activities of the GCS seen in our model.

In summary, we could establish an *ex vivo* mucosal model to study allergen and rhIL-1 β -induced E-selectin expression in nasal allergic mucosa. Both allergen and IL-1 β stimulation led to a significant upregulation of E-selectin on endothelial cells within 1–2 h. A short incubation time of only 30 min before allergen exposure with different GCS almost completely inhibited E-selectin expression, whereas stimulation with rhIL-1 β was only partially inhibited by fluticasone, which showed the strongest activity of the GCS tested in the allergen-induced setting. These findings suggest the prominent role of an indirect action on adhesion molecule expression by interference with the release of proinflammatory cytokines. In this model, fluticasone propionate was more active than prednisolone and flucortin butyl, indicating that this *ex vivo* mucosal model may represent a valid means to compare activities of different GCS.

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