

IgE-Dependent and IgE-Independent Stimulation of Human Basophils Increases the Presence of Immature FcεRIα by Reversing Degradative Pathways

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Key Words

IgE · IgE receptor · Receptor processing

Abstract

Background: Expression of the high-affinity IgE receptor, FcεRI, on mast cells and basophils has previously been shown to be sensitive to the presence of IgE or cytokines. The current study examined whether stimulation of human basophils resulted in a change in the expression of FcεRI. **Methods:** Changes in the well-expressed immature intracellular form of the receptor, FcεRIα (p46), were examined by quantitative PCR, Western blot and the pulse-chase method. **Results:** Both IgE-dependent (anti-IgE antibody) and IgE-independent stimulation [formyl-methionyl-leucyl-phenylalanine (FMLP) and C5a] led to increased accumulation of p46. The p46 form of FcεRIα increased 1.52 ± 0.09 -, 2.58 ± 0.09 - and 1.47 ± 0.07 -fold following stimulation with anti-IgE, FMLP and C5a, respectively. There were no changes in the steady-state levels of mRNA for FcεRIα. The kinetics of the increase in p46 was slow following stimulation with anti-IgE antibody, with the earliest increases observed after 8 h. The p46 form was degraded in a bafilomycin A (lysosomal inhibitor)-sensitive process. There was no synergy between treatment with bafilomycin A and anti-IgE or FMLP stimula-

tion, suggesting that the 2 methods of enhancement operate on the same pathway. Pulse-chase studies corroborated this conclusion. In contrast, IL-3 and bafilomycin A synergistically increased p46, suggesting that IL-3 increased synthesis of FcεRIα. **Conclusions:** Taken together, these results suggest that secretagogue stimulation results in an increase in p46 due to reversal of degradative pathways rather than increased synthesis of FcεRIα. Nevertheless, a decrease in the degradation of FcεRIα at an intermediate step in its processing by non-FcεRI-dependent stimulation may still influence expression of this important receptor.

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Introduction

Expression of the high-affinity IgE receptor on mast cells and basophils allows these cells to interact with environmental substances through cell-bound IgE to initiate secretion of inflammatory mediators such as histamine. Cell surface expression of FcεRI is a steady-state process, with the rate of synthesis and loss of unoccupied receptors regulated by different processes. IgE antibody inhibits the loss of unoccupied receptors from the cell surface [1, 2], and various cytokines influence its synthe-

sis [3–6]. There appear to be multiple mechanisms by which generation of FcεRI is influenced. For example, in murine mast cells, IL-10 alters protein synthesis, but the presence of mRNA for the receptor subunits is not altered [7]. There have also been indications that stimulation of human basophils increased the presence of FcεRIα mRNA transcripts [8].

In rodent mast cells, aggregation of FcεRI induces its rapid loss from the cell surface [9]. In human basophils, the same process occurs but requires many hours to days to achieve [10]. Nevertheless, with respect to cell surface expression, the most noticeable effect of receptor aggregation is downregulation in both murine and human cells. It is possible that to compensate for this loss, aggregation results in a change in the receptor synthesis rate. This could result from internal events initiated by receptor aggregation or an autocrine loop that results from newly generated cytokines feeding back to the cell to alter expression or synthesis. However, the downregulation of aggregated cell surface receptors complicates analysis of any potential changes in cell surface receptor expression. We have previously noted that human basophils normally express 2 variants of the alpha subunit of FcεRI, termed p60 and p46 [11]. The p60 form is heterogeneously glycosylated and expressed on the cell surface. The p46 form represents a pre-Golgi form (most likely located in the endoplasmic reticulum) [12–16] whose glycosylation is sensitive to endoglycosidase H [11]. A survey of the general population shows that expression of p60 is highly variable and consistent with the known sensitivity of the receptor to serum IgE levels. For example, surface expression ranges from 5,000 to 500,000 receptors per basophil. In contrast, the expression of the p46 form is relatively constant in the general population, being present at a level that is estimated to represent approximately 100,000–200,000 receptors per cell. There is no evidence that this form of the receptor is sensitive to the aggregation of cell surface receptors [10]. Therefore, its relative presence may act as a sensor of changes in the generation of FcεRIα without the complication of downregulatory events on the cell surface. Furthermore, the high levels of p46 present in freshly isolated basophils rapidly diminish during culture. It is unclear what modulates this change and it is unclear what role this large amount of internal FcεRIα plays in basophil biology. These studies began with a simple question: Would stimulation through FcεRI induce changes in the presence of p46 or, more specifically, an upregulation of its presence? However, these studies were also initiated in order to develop some understanding about the factors that influence p46 levels.

Materials and Methods

Materials

The following materials were purchased from the indicated sources: PIPES, bovine serum albumin (BSA), EDTA, D-glucose, NaF, Na₄P₂O₇, Na₃VO₄, 2-mercaptoethanol, NP-40 and formyl-methionyl-leucyl-phenylalanine (FMLP) from Sigma, St. Louis, Mo., USA; crystallized human serum albumin (HSA) from Miles Laboratories, Elkhart, Ind., USA; fetal calf serum and RPMI-1640 containing 25 mM HEPES and L-glutamine from BioWhittaker, Walkersville, Md., USA; Percoll from Pharmacia, Piscataway, N.J., USA; tris (hydroxymethyl)-aminomethane and Tween-20 from Bio-Rad, Hercules, Calif., USA; leupeptin, dithiothreitol and 4-(2-aminoethyl)benzene sulfonyl fluoride (AEBSF) from Sigma-Aldrich, St. Louis, Mo., USA; mouse anti-FcεRIα monoclonal antibody 22E7 was a gift from Hoffman-La Roche, Nutley, N.J., USA; horseradish peroxidase (HRP)-conjugated donkey anti-rabbit Ig antibody, HRP-conjugated sheep anti-mouse Ig antibody and protein G sepharose beads from Amersham Life Sciences, Arlington Heights, Ill., USA; FMLP, actinomycin D and cycloheximide from Sigma-Aldrich, and MG-132, lactacystin A and proteasome inhibitor I from EMD Biosciences, Gibbstown, N.J., USA.

Buffers

PIPES-albumin-glucose (PAG) buffer consisted of 25 mM PIPES, 110 mM NaCl, 5 mM KCl, 0.1% glucose and 0.003% HSA, pH 7.4. Other buffers used were PAG supplemented with 1 mM CaCl₂ and 1 mM MgCl₂, as well as PAG supplemented with 4 mM EDTA. Countercurrent elutriation was conducted in PAG containing 0.25% BSA in place of 0.003% HSA. For electrophoresis, Novex electrophoresis sample buffer containing 5% 2-mercaptoethanol was used. Complete lysis buffer was 20 mM Tris-HCl, pH 7.5, 100 μg/ml aprotinin, 10 mM benzamidin, 1 mM AEBSF, 100 μg/ml leupeptin, 50 mM NaF, 1 mM Na₃VO₄, 1% NP-40 and 10% glycerol. Incomplete lysis buffer was complete lysis buffer without the protease inhibitors, NP-40, glycerol or vanadate.

Basophil Purification

Residual cells from normal donors were subjected to leukapheresis and enriched in basophils using a combination of Percoll density gradients and countercurrent flow elutriation, as described previously [17]. The cells were further purified by negative selection using a cocktail of antibodies from Stem Cell Technologies (Vancouver, B.C., Canada; basophil purification kit) and columns from Miltenyi (Auburn, Calif., USA). The purity of the basophils was determined by alcian blue staining [18], and basophils purified from leukapheresis packs generally exceeded 99% purity.

Basophil Cultures

Purified basophils were cultured at 2×10^6 /ml in RPMI-1640 medium supplemented with 10 μg/ml gentamicin, 300 μg/ml HSA and 1 mM CaCl₂ with or without IL-3 (10 ng/ml) as described in Results. In some experiments, cells were incubated with cycloheximide, actinomycin D, bafilomycin A or MG-132 or stimulated with FMLP, C5a or anti-IgE antibody. Several parameters of cell viability were investigated for some experiments, i.e. recovery, erythrocin B positivity and annexin V + propidium iodide positivity (by flow cytometry).

Western Blots

Purified basophils (approximately $0.5\text{--}1 \times 10^6$ depending on the condition) were pelleted and lysed in 20 μl of electrophoresis sample buffer, then the tube was placed in boiling water for 5 min and the samples were stored at -80°C until electrophoresis. Samples were run in a 15-well 10% Tris-glycine gel with molecular weight markers, and after transfer and blocking with 4% BSA, the nitrocellulose was incubated with 22E7 (mouse anti-Fc ϵ RI α). Bands were visualized with HRP-conjugated anti-mouse antibody, treated with enhanced chemiluminescence reagent for detection by film. Film exposures were digitized, and the digital images were analyzed with NIH Image. Following the 22E7 blot, the nitrocellulose was blotted again, without stripping, with an antibody to the p85 α regulatory subunit of phosphoinositide 3-kinase. This protein is well expressed in basophils and has been shown not to vary during culture or stimulation [19]. It therefore acts as a better internal standard for lane normalization than actin, since the p46 band of Fc ϵ RI α migrates in the same general region as actin.

Real-Time PCR

RNA was isolated by lysing purified basophils (2×10^6) and processing lysates with the Roche High Pure RNA Isolation kit (Roche Applied Science, Indianapolis, Ind., USA). Forward and reverse oligo primers for Fc ϵ RI α were constructed using Primer Express software and previously published cDNA sequences [1]. For Fc ϵ RI α , the forward primer was 5'-GTGAACCTGTGTACCTGG-AAGTCTT-3' (nucleotide position 408, Genbank accession number X06948) and the reverse primer was 5'-CATCCCAGTTCCTCCAACCA-3' (position 524). The probe sequence for Fc ϵ RI α was 5'-TGACTGGCTGCTCCTTCAGGCCTC-3'. The primers for IL-4 were 5'-CGACTGCACAGCAGTTCCA-3' and 5'-CAGG-CCCCAGAGGTTC-3', and the probe was 5'-TCCGATTCC-TGAAACGGCTCGACA-3'. For all probes, the 5' reporter dye was FAM and the 3' quencher dye was TAMRA. The real-time PCR method is based on the cleavage of fluorescent dye-labeled probes by the 5'-3' exonuclease activity of the *Taq* DNA polymerase during PCR and measurement of fluorescence intensity by a Sequence Detection System (Perkin Elmer, 7700).

Pulse-Chase Studies

Basophils were firstly cultured for 24 h in RPMI containing no unlabeled methionine but supplemented with 0.5 μM ^{35}S -methionine in order to load the p46 compartment. The cells were transferred to normal RPMI without ^{35}S -methionine, cultured for 2, 4 or 24 h and lysed. Then the p46 form of Fc ϵ RI α was immunoadsorbed with anti-Fc ϵ RI α antibody (Upstate Biologicals Inc.), run on 10% gels and transferred to nitrocellulose for radiography. For some of these studies, bafilomycin A was used at 0.6–2.0 μM in the chase period, concentrations that would be too high for longer periods but were considered acceptable for the short 2- to 4-hour chase period. Calculation of reversal was performed using the following equation: $100 \times [1 - (\text{BD}_0 - \text{BD}_t)/(\text{BD}_0 - \text{BD}_c)]$, where BD = radiometric band density, BD_0 = density at time zero (beginning of chase), BD_t = density at 4 h for treated sample and BD_c = density at 4 h for vehicle control sample.

Statistics

For many of the experiments, the data are presented as a stimulation index, i.e. the ratio of stimulation to no stimulation on

cells recovered on day 1. The precise numerator and denominator for the calculation depended on the experimental design (see Results). Data are presented as means $\pm$ standard error of the mean (SEM). For most of the studies, the null hypothesis was a stimulation index of 1.0 (no effect). ANOVA was used to determine statistical significance for the synergy experiments.

Results

In vitro Induced Changes in p46: Stimulation

Changes in p46 were examined after basophils were stimulated with anti-IgE antibody. The initial fundamental question was whether IgE-mediated stimulation would modify the synthesis of Fc ϵ RI α , which would be manifested at early time points in the appearance of p46. The presence of p46 after 24 h of culture with and without stimulation with anti-IgE antibody at an optimal concentration (0.2 $\mu\text{g}/\text{ml}$) was compared. As shown in figure 1a and b, the presence of anti-IgE antibody did significantly increase the steady-state level of p46 (relative to no stimulation) [for examples of the loss of cell surface p60 during the same stimulation, see ref. 10]. These experiments were performed in the absence of IL-3. Figure 1c shows that the kinetics of the increase in p46 was slow, with little or no increase occurring before 8 h.

Using quantitative PCR to measure Fc ϵ RI α mRNA [1], we found that stimulation with anti-IgE antibody did not increase the presence of mRNA for Fc ϵ RI α at 1, 2, 4, 24 or 48 h (fig. 2). As a positive control, IL-4 mRNA at the 1- and 2-hour time points was elevated, indicating effective stimulation and mRNA recovery.

The absence of a change in the Fc ϵ RI α mRNA steady state raised questions about what was occurring. We firstly asked whether any form of stimulation increased the presence of p46. Figure 1a and b shows that stimulation with FMLP and C5a also significantly increased the level of p46, but notably, the efficacy of enhancement was greater for FMLP than for anti-IgE antibody or C5a. FMLP did not alter the presence of p60 (cell surface Fc ϵ RI α); the ratio of p60 expression with FMLP to that without FMLP after 18–24 h of culture was 0.90 ± 0.09 ($n = 7$; not significant). In addition, no changes in the numbers of basophils were noted in these 24-hour cultures, as evidenced by the constancy of expression of the control protein, p85 (regulatory subunit of phosphoinositide 3-kinase).

In vitro Induced Changes in p46: Steady-State Issues

Based on studies in both rodent and human mast cells and basophils, the model of p46 regulation shown in figure 3 is suggested [10–14, 20–26]. In this scheme, after

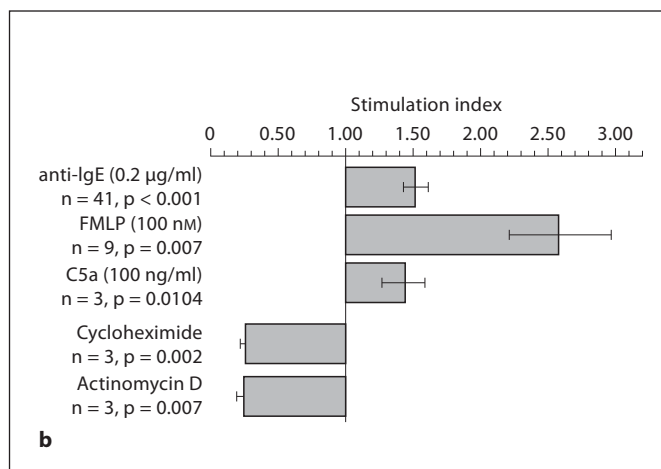
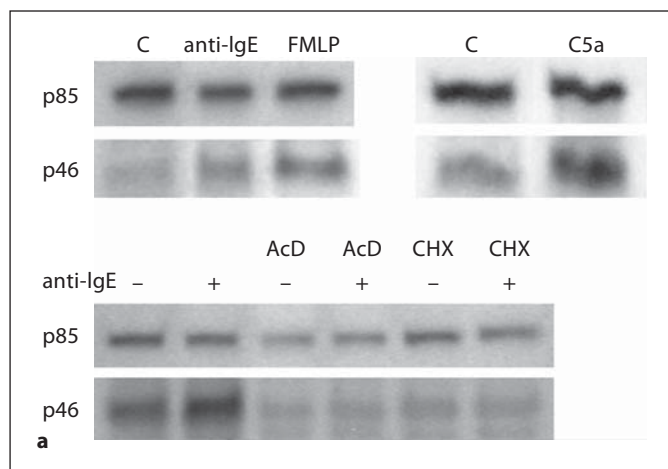
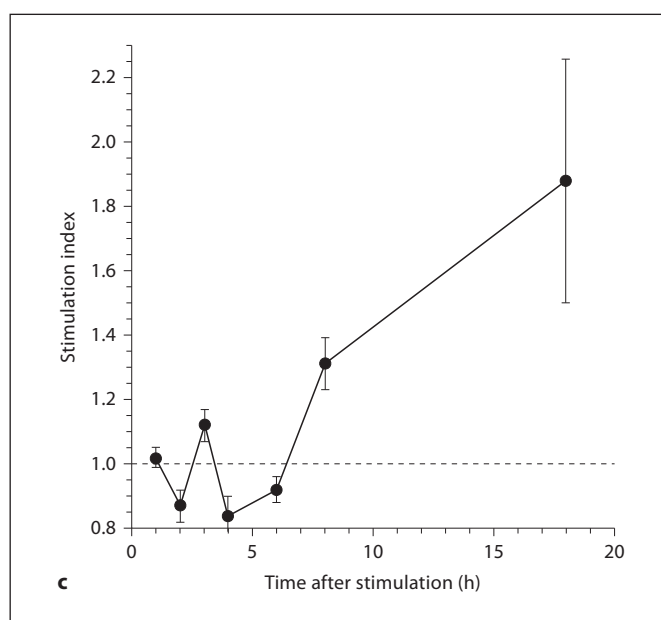


Fig. 1. IgE-mediated regulation of p46 FcεRIα expression in basophils. Purified basophils were cultured under various conditions for 24 h, i.e. vehicle control (C), where the vehicle was DMSO if cycloheximide or actinomycin D were used and buffer alone for stimuli, anti-IgE at 0.2 µg/ml, FMLP at 100 nM, C5a at 100 ng/ml, cycloheximide (CHX) at 10 µM and actinomycin D (AcD) at 5 µM. **a** Example Western blots for basophils cultured for 1 day with and without the indicated stimuli or drugs. p46: immature FcεRIα; p85: the lane loading control protein (see Methods). **b** The ratio of p46 band densities from Western blots shown in **a** for various conditions. Statistical p values for H_0 ratio = 1.0 are shown. The band densities are normalized to p85 band densities (see Methods). **c** The kinetics of the increase in p46 levels (relative to unstimulated control) following stimulation with 0.2 µg/ml anti-IgE antibody (n = 3).



synthesis, p46 undergoes further processing, i.e. either loss by degradation or reprocessing through the Golgi to generate p60 (cell surface FcεRIα). As such, p46 represents a steady state with 1 inflow and at least 2 outflow paths. The synthesis step was initially examined by inclusion of cycloheximide or actinomycin D. Both agents nearly ablated the presence of p46 (fig. 1a, b).

As noted previously, p46 levels in freshly isolated basophils are quite significant [11], but the first overnight culture reduces levels by 80–90% [11]. This was evidence of an active degradative process for p46, and this led to the hypothesis that accumulation of p46 following stimulation may be a result of a decreased rate of degradation. We were therefore interested in whether blocking a deg-

radative pathway, which is one of the outflow paths shown in the model, would synergize with the effect of stimulation or have no additional effect. There are 2 well-studied mechanisms of degradation of proteins in cells, namely, loss through proteasomes and loss through lysosomes. As shown in figure 4a, MG-132, a proteasome inhibitor, ablates p46 in overnight culture. However, all the proteasome inhibitors we tested induce significant cell death in an 18- to 24-hour time frame. For example, incubating purified basophils in RPMI media containing IL-3 resulted in $90 \pm 3\%$ viability after 24 h and $64 \pm 9\%$ viability in the presence of 5 µM MG-132 ($p = 0.023$ compared to control; n = 5; lactacystin A and proteasome inhibitor I result in a similar loss of viability). The ablation of p46

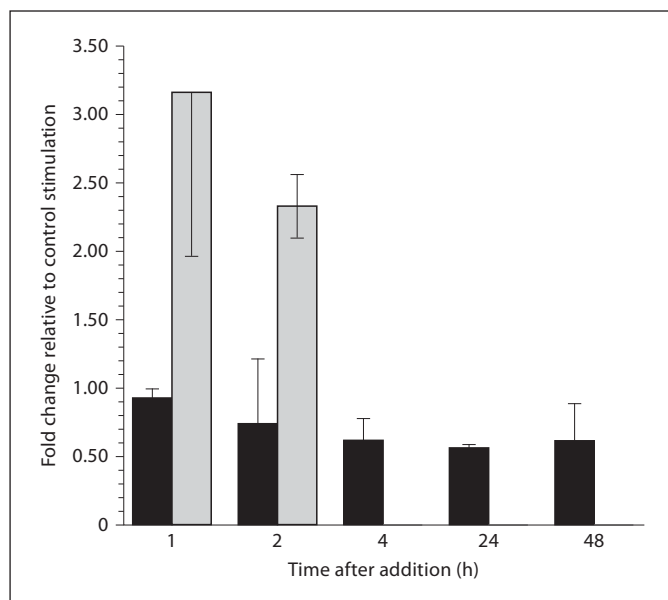


Fig. 2. Expression of FcεRIα mRNA in basophils stimulated with anti-IgE antibody. Purified basophils were stimulated with an optimal concentration of anti-IgE antibody (0.2 μg/ml) in medium and cultured for the times shown (n = 2). The isolated mRNA was analyzed by quantitative PCR, and the data are expressed relative to cells that were not stimulated for the same time periods. To provide evidence that the cells experienced stimulation, IL-4 mRNA was also assessed at the 2- and 4-hour time points (IL-4 mRNA is known to decay to resting levels within several hours, so later time points would not have been informative). Black bars: FcεRIα mRNA; gray bars: IL-4 mRNA.

expression is not expected; either there should be no effect (if degradation is lysosomal-dependent and not proteasomal-dependent) or p46 should accumulate (if there is a component of proteasomal degradation). These observations suggest that results using agents that induce apoptosis cannot be readily interpreted. Note also the relatively lower expression of the control protein, p85, in the Western blots (fig. 1a) shown after actinomycin D treatment (both with and without anti-IgE antibody) and to a lesser extent with cycloheximide, suggesting poorer recovery of basophils in these cultures.

Previous studies demonstrated that bafilomycin A (an inhibitor of lysosome-mediated protein degradation) inhibits degradation of p60 when it is internalized as a result of aggregation or lack of occupancy [10]. Therefore, the effect of 200 nM bafilomycin A on the levels of p46, with or without IL-3, was examined. Previous studies [10] have shown that bafilomycin A concentrations greater than 1.0 μM have, at best, modest effects on basophil vi-

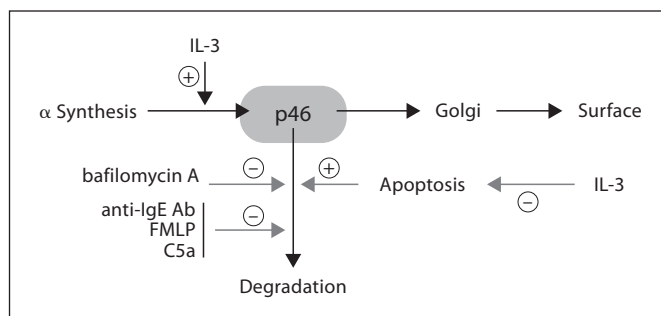


Fig. 3. Working model for the regulation of p46 FcεRIα expression in basophils. After synthesis of immature p46, FcεRIα is either transported to Golgi for further processing and expression on the cell surface (p60 FcεRIα) or remains in the endoplasmic reticulum to eventually undergo bafilomycin A-sensitive degradation. The influences on the various pathways explored in this study are shown. Ab = Antibody.

ability for times greater than 18 h but that a 200 nM concentration provides a balance between effective inhibition of degradation and no apparent effects on viability. Figure 4b shows that there was an increased presence of p46 with the inclusion of bafilomycin A.

These initial experiments were performed without IL-3, an agent that has been proposed to alter FcεRIα synthesis [1]. Therefore, the effects of IL-3 were examined next. Firstly, comparing day 1 to day 0 (preculture) levels of p46 in cultures without IL-3, there was some loss of p46 relative to the level of p46 before incubation (ratio of day 1 to day 0 levels 0.56 ± 0.11 ; n = 7; p = 0.004). Figure 4c shows that compared to overnight incubation without IL-3, incubation with IL-3 increased the presence of p46 approximately 1.6-fold, although the effect was quite variable. However, this effect essentially reversed the loss that occurred naturally without IL-3 (ratio of day 1 to day 0 levels with IL-3 0.79 ± 0.39 ; n = 9; not significant for $H_0 = 1.0$).

In the presence of both bafilomycin A and IL-3, there was a marked increase in steady-state levels of p46 (fig. 4c), although the increase was highly variable (fig. 4 shows dot plots in order to clearly show the broad range of stimulation indices in some of these experiments). In some instances, bafilomycin A only slowed the natural loss of p46, and in other instances, it led to more p46 than was present on day 0. This result suggested that p46 was being degraded by a bafilomycin A-sensitive lysosomal pathway.

Given that our model includes 2 outflow paths, differences in the fractions of p46 that are directed into the 2

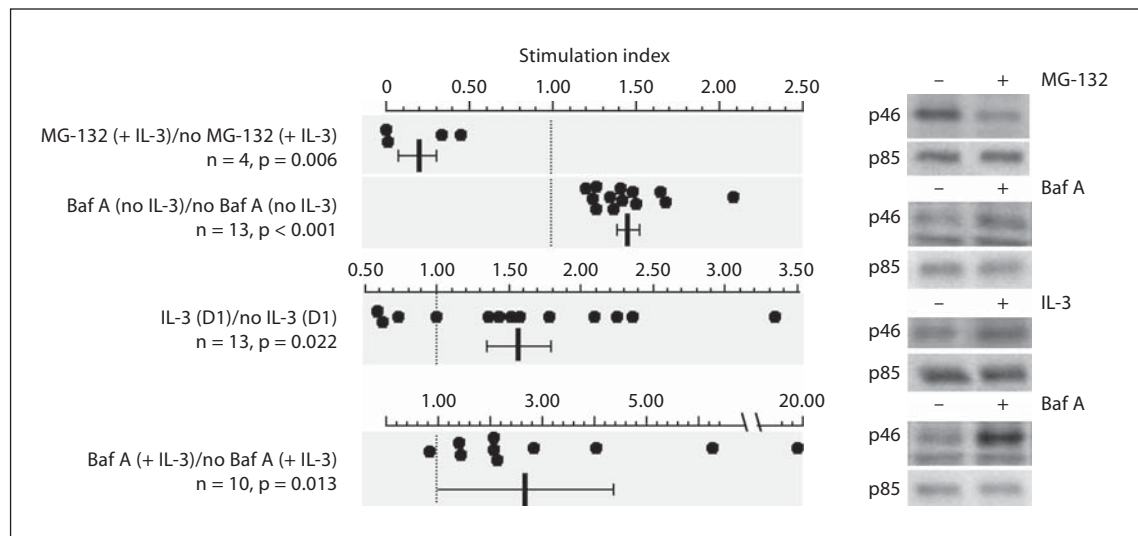


Fig. 4. Effects of proteasome and lysosome inhibitors on p46 FcεRIα expression. Purified basophils were cultured under various conditions for 24 h. The compounds were used at the following concentrations: MG-132 at 5 μM, bafilomycin A (Baf A) at 200 nM and IL-3 at 10 ng/ml. The stimulation index of p46 band densities from Western blots for various conditions is plotted and the

statistical p values for H_0 ratio = 1.0 are shown. For the purpose of showing the broad range of behavior in these experiments, each dot represents the results from a different preparation of basophils, with the mean and SEM shown for the group. D1 = Day 1. In the right panels, example Western blots for each experiment are shown (the p85 lane loading controls are also shown).

pathways might be expected to produce considerable variability in the ability of bafilomycin A to alter p46 levels. With this in mind, we examined the effects of brefeldin A to block the outflow pathway leading to Golgi reprocessing. However, we found that this agent significantly reduced p46 and led to loss of cells. In combination with bafilomycin A (to inhibit both outflow pathways), the loss of p46 was greater and cell loss was also greater (data not shown). Therefore, it was not possible to interpret the results for brefeldin A.

With the model in figure 3 in mind, it can be seen that stimulation (anti-IgE, FMLP or C5a) might not result from increased synthesis of p46 but decreased outflow, through shunting of p46 either to degradation or to the Golgi apparatus. If stimulation inhibited loss, then inclusion of bafilomycin A would not be expected to synergize with the effect of stimulation, since both would operate on the same outflow pathway. In practice, a small increase with the combination might be expected, since we adjusted the bafilomycin A concentration to be less than maximally effective in order to reduce collateral effects on apoptosis during an 18- to 24-hour culture. The results of this experimental design depend on the stimulus used. Figure 4a and b shows that there was no synergy if the stimulus was either FMLP or anti-IgE antibody. In

other words, if synergy were to occur because stimulation enhanced synthesis and bafilomycin A inhibited degradation, then the expectation (assuming a multiplier effect) was for an increase, as shown in the fourth column of figure 5a–c. For neither stimulus did the combination of agents lead to a statistically significant increase relative to bafilomycin A alone. However, if IL-3 is considered a ‘stimulus’, then there was synergy (fig. 5c; ANOVA: overall p = 0.023; for the difference between IL-3 and IL-3 + bafilomycin A, p = 0.013; for the difference between bafilomycin A and IL-3 + bafilomycin A, p = 0.032).

If bafilomycin A blocks one of the arms of the steady-state outflow, leading to accumulation of p46 greater than the starting level of p46, the results further suggest that p46 is being constantly synthesized in culture. To circumvent the issues raised by the use of cycloheximide or actinomycin D, basophils were cultured in the presence of ^{35}S -methionine. After 24 h of labeling, the cultures were washed free of ^{35}S -methionine for a chase period of 2, 4 or 24 h. During the chase period in some experiments, cells were treated with bafilomycin A. Lysates of basophils were immunoadsorbed for FcεRI (p60 and p46). In all cases, there was a radiometric band at p46 after the labeling period. The specific activity is unknown,

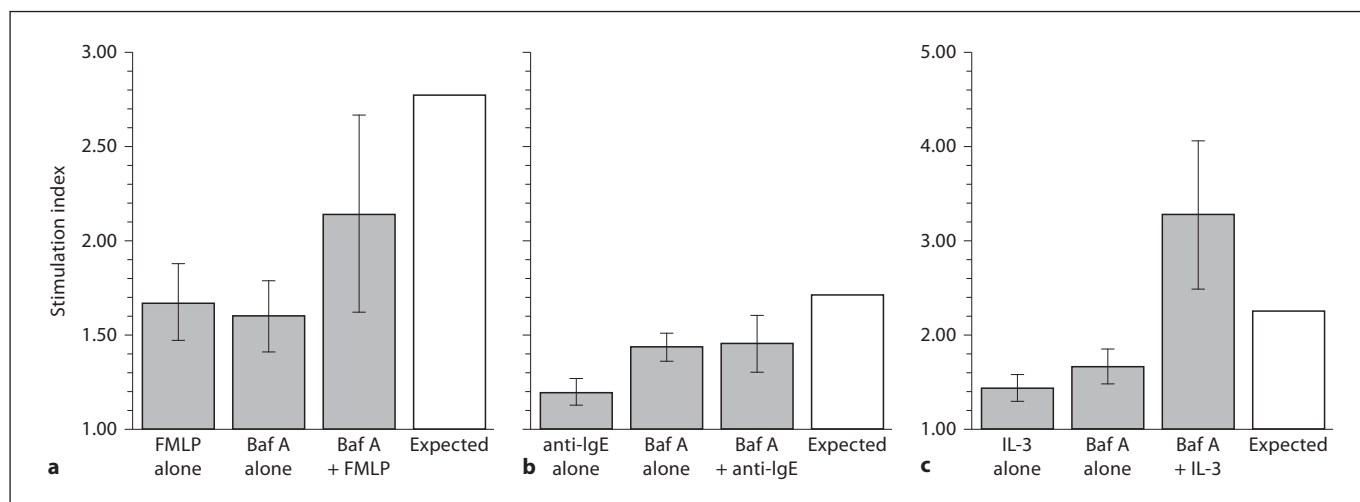


Fig. 5. Test for synergy between treatment with bafilomycin A and stimulation. **a** Purified basophils were cultured under various conditions for 24 h, i.e. stimulation with FMLP (100 nM) alone, bafilomycin A (Baf A; 200 nM) alone or bafilomycin A (200 nM) plus FMLP (100 nM). The stimulation index was calculated as the ratio of band densities from Western blots for the various conditions to basophils cultured without the agents (vehicle control; $n = 4$). The column labeled 'expected' represents the multiplica-

tion of stimulation indices for FMLP alone and bafilomycin A alone. **b** Using the experimental design described for **a**, p46 levels were examined after stimulation with anti-IgE antibody (0.2 $\mu\text{g/ml}$), bafilomycin A (200 nM) alone or bafilomycin A (200 nM) plus anti-IgE (0.2 $\mu\text{g/ml}$) ($n = 6$). **c** Using the experimental design described for **a**, p46 levels were examined after stimulation with IL-3 (10 ng/ml), bafilomycin A (200 nM) alone or bafilomycin A (200 nM) plus IL-3 (10 ng/ml) ($n = 7$).

but this indicated that there is ongoing synthesis of p46. Figure 6a shows the chase kinetics, showing a half-life of approximately 4 h and indicating an ongoing, relatively rapid loss of p46. Changes in the loss during the chase period were examined. The ability of bafilomycin A or FMLP to reverse the loss was highly variable (fig. 6b), a variability similar to that found with mass measurements, with 5 of 10 preparations showing some reversal but the remaining preparations showing no reversal. Therefore, in aggregate, there was not a statistically significant alteration. However, there was concordance between the ability of bafilomycin A and FMLP to reverse loss (fig. 6c; $r = 0.97$, $p < 0.0001$). The tentative interpretation of these experiments is that stimulation inhibits degradation. In 2 experiments, the effect of IL-3 on the chase period was also examined, and IL-3 was found not to change the rate of loss (data not shown).

The data suggest that receptor synthesis is slow in cultured basophils, and while IL-3 appeared to augment synthesis, 24 h was insufficient to observe large changes. Therefore, basophils were cultured for 13 days in IL-3, and under these conditions, p46 levels increased above preincubation levels, on average 7.7-fold (range 3.2- to 35-fold, $n = 5$).

Discussion

IgE-mediated stimulation of human basophils results in a slow loss of cell surface Fc ϵ RI. Although far slower than often found in other models of receptor downregulation, the process would nevertheless disguise the generation of new receptors that might occur in response to stimulation as a compensatory mechanism for receptor loss. Consequently, we examined the behavior of the immature form of Fc ϵ RI α , p46, for which there have been few previous experimental studies in basophils and which might act as a surrogate measure of changes in the generation of Fc ϵ RI α . Previous studies have suggested that ongoing receptor synthesis in cultured peripheral blood basophils is a slow process. Therefore, detection of new receptors on the cell surface was anticipated to be technically difficult. This expectation was supported by the ^{35}S -methionine labeling studies. We were able to show accumulation of newly synthesized receptors by this method, but the signals were not strong enough to readily observe their appearance on the cell surface. However, they were strong enough to show accumulation of the immature form and to carry out pulse-chase studies. These results also suggested the strategy of examining p46 as a sensor of the process of

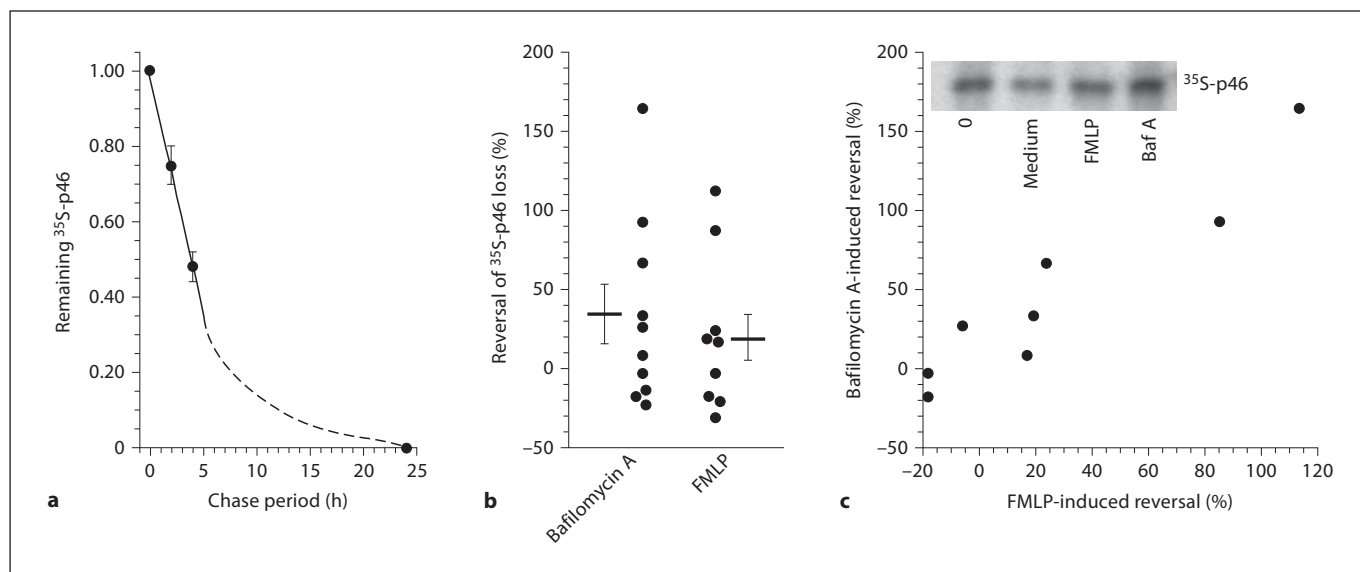


Fig. 6. Pulse-chase study. Basophils were cultured in methionine-free RPMI-1640 supplemented with ^{35}S -methionine for 20 h (pulse), followed by incubation in normal RPMI-1640 with vehicle for 0, 2, 4 and 24 h (chase) (see Methods). **a** Kinetics of the loss of labeled p46 without any agents added. p46 levels are shown as a fraction of the level present before incubation. **b** Effect of bafilomycin A (2 μM) or FMLP (100 nM) for a 4-hour chase period relative to time zero (start of chase period) and vehicle-control cells

at 4 h (see Methods for reversal calculation). Each dot represents the results from a different preparation of basophils, with the mean $\pm$ SEM shown for each group. **c** Where bafilomycin A and FMLP were tested in the same experiment, the relative reversal for both is plotted. Inset: a radiometric image of the ^{35}S -labeled p46 band from 1 experiment where medium alone, 100 nM FMLP or 2 μM bafilomycin A (Baf A) was added for a 2-hour chase period. 0 = Day 0.

Fc ϵ RI α generation. However, because p46 is an intermediate intracellular form of Fc ϵ RI α , and because it is apparent that p46 accumulates in human basophils *in vivo* [11], it is necessary to consider its study as a steady-state problem. The model shown in figure 3 captures these considerations and studies performed in other cells [10–14, 20–26].

The preliminary studies of unlabeled Fc ϵ RI α p46 demonstrated changes in its presence following stimulation with anti-IgE antibody. The process was slow, requiring many hours to fully develop in these *in vitro* cultures. As noted in the Introduction, a previous study [8] found increased transcript for Fc ϵ RI α (and Fc ϵ RI β) after stimulation via Fc ϵ RI. The increase in that study was modest (approx. 2-fold) and was determined by microarray analysis. However, in our study, stimulation with anti-IgE antibody was not accompanied by changes in the steady-state levels of mRNA for Fc ϵ RI α . In a more recent but unrelated study, we examined stimulation of human basophils with anti-IgE antibody at 2 h using a gene expression microarray. In our array results, there was no change in mRNA expression for FcR β or FcR γ . At this time, we do not understand the reason for the difference between the published results and our experience with

respect to changes in mRNA levels. It might be related to the strength of stimulation in the particular cells examined, although there is no information regarding this parameter. One speculative possibility is that if the basophils generate enough IL-3 [27], then IL-3 could feed back to induce receptor mRNA increases [28]. There are 2 problems with this speculation. Firstly, in the study of Youssef et al. [8], the increases in Fc ϵ RI α and FcR β mRNA only occurred if the cells were washed 30 min after stimulation (which we also did in our microarray studies but not for the experiments shown in fig. 2). If IL-3 is necessary to observe the effect, then it must be produced after washing (which is appropriate [27]) and the washing must remove some inhibitory factors. Secondly, FMLP does not induce IL-3 production [27], and we continued to observe an increase in Fc ϵ RI α with FMLP. Finally, the study by Youssef et al. [8] did not examine increased expression of Fc ϵ RI α or FcR β protein, so it is not clear that the mRNA results of that study actually translated to increased protein expression.

We also found that non-IgE-dependent stimulation also led to the accumulation of the p46 form of Fc ϵ RI α . There was a marked loss of p46 when cells were treated

with actinomycin D or cycloheximide, supporting the idea of a synthesis process being necessary for p46 accumulation. However, results with MG-132 raised concern about what was occurring during conditions in which apoptosis might also be occurring. It was not expected that treatment with MG-132, a proteasome inhibitor, would lead to marked loss of p46. It does not induce this change in surface FcεRIα [10]. We found that MG-132 and other proteasome inhibitors induced significant apoptosis of basophils cultured for only 18–24 h. This result led to the conclusion that results using agents that altered apoptosis would be difficult to interpret. Although 18 h of treatment of basophils with cycloheximide does not appear to cause changes in viability [29], incubation with protein synthesis or transcriptional inhibition reverse the effects of IL-3 [29], and IL-3 promotes survival in basophils [28, 30]. In light of this, the actinomycin D and cycloheximide data should be interpreted cautiously.

These concerns raised the possibility that the changes being observed were related to changes in the degradation of p46 rather than changes in synthesis and that some caution should be observed when using agents that alter survival of basophils. When comparing day 1 to day 0, IL-3 did reverse a loss of p46 that occurred in cultures where IL-3 was not present. Indeed, the effect of anti-IgE antibody was similar in this regard. Only FMLP generally increased the amount of p46 on day 1 relative to day 0. Significant accumulation above the day 0 levels also occurred with IL-3 and bafilomycin A. These data suggest that the primary effect of stimulation is to reverse the loss/degradation of p46.

Although stimulation appeared to reverse the loss of p46 to levels similar to the effect of IL-3, there was no evidence that stimulation acted in a manner that mimicked the effects of IL-3 on survival. In particular, there were no changes in cell number when cells were cultured with stimulus in the absence of IL-3. Furthermore, there was synergy between bafilomycin A and IL-3 that did not occur with stimulation. From the perspective of the model shown in figure 3, enhancement of synthesis and inhibition of loss (through degradation) should synergistically enhance the presence of p46. However, simultaneous inhibition of loss by 2 methods that operate on the same outflow pathway would look similar to either inhibitory method alone (provided inhibition of degradation was near maximal in either case). Neither FMLP plus bafilomycin A nor anti-IgE antibody plus bafilomycin A significantly enhanced the presence of p46 beyond the effect of bafilomycin A alone. In contrast, IL-3 and bafilomycin A were synergistic in their effects.

If stimulation inhibited degradation, it should have been possible to observe a change in the rate of loss of ³⁵S-labeled p46. Although the average rate of loss in the pulse-chase experiments was slower in the presence of bafilomycin-A or FMLP, the difference was not statistically significant. However, it is perhaps notable that there was strong concordance between inhibition of loss induced by FMLP and bafilomycin A, suggesting that when they do work in a particular cell culture, they operate on the same pathway. It is not clear why the inhibitory effect (on the loss of labeled p46) was not more consistent, but this inconsistency was noted in the unlabeled studies as well. Considering that there are 2 outflow pathways shown in the model in figure 3, one of which we could not effectively inhibit, and considering the biological variability between subject basophils and the potential confounding effects of IL-3, it may not be surprising that there was considerable variation in the response to bafilomycin A or FMLP.

Taken together, these studies suggest that stimulation with anti-IgE antibody, FMLP or C5a induces a significant increase in the presence of the immature form of FcεRIα, p46, but that this increase results from a change in the degradative processing of p46 rather than an increase in synthesis. The most efficient reversal of this loss occurred with FMLP, with anti-IgE antibody and C5a only partially slowing loss. The molecular mechanism(s) underlying this ability of stimulation to alter the processing pathways (vs. synthesis pathways) of p46 is/are unknown, but the bafilomycin A experiments indicate that degradation follows a lysosomal pathway. Although a change in synthesis did not appear to occur, the changes in p46, which reflect an intermediate stage in the pathway to surface expression, were significant, 2.5-fold in the case of FMLP. Such a change might influence the efficiency with which FcεRIα is translated to the cell surface and, therefore, alter expression of this important receptor on basophils, even in cases where stimulation occurs through non-FcεRI receptors. These studies also demonstrated the influence of IL-3 on p46 accumulation. In conjunction with the new information about regulation of degradation, the results with IL-3 support previous studies suggesting that IL-3 enhances synthesis of this receptor. In summary, these results suggest that the high levels of p46 observed in fresh peripheral blood basophils might reflect the overall stimulatory and cytokine environment of these cells in vivo. The data suggest that for FcεRIα to be translated to the cell surface, there are extrinsic determinants that control an intermediate stage of processing that occurs before the protein enters the Golgi apparatus.

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