Antigen-induced reduction in mast cell and basophil functional responses due to reduced Syk protein levels

By: Christopher Kepley

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Abstract:

Background: The high-affinity IgE receptor, FcεRI, is unresponsive on mast cells and basophils from people in several populations through an unknown mechanism. Similarly, FcεRI-positive basophils from 'nonreleasers' are IgE-unresponsive and are deficient in the tyrosine kinase Syk. *Objective:* To test the hypothesis that cross-linking FcεRI on mast cells and basophils leads to FcεRI nonresponsiveness through reduction in Syk protein levels. *Methods:* Human mast cells and basophils were used to determine if FcεRI hyporesponsiveness correlated with reduced Syk levels. *Results:* It is shown that suboptimal antigen challenge, that did not lead to significant mediator release, induced nonresponsiveness and correlated with reduced Syk. Other IgE-associated signaling molecules were unaffected by the same treatment. The ability of IgE-unresponsive mast cells to regain FcεRI responsiveness is paralleled by increased cellular Syk levels in vitro. The reduction of Syk levels with suboptimal antigen concentrations was calcium independent and mediated through a proteasome-dependent mechanism. *Conclusion:* These findings confirm and extend our knowledge about a novel regulatory mechanism for maintaining FcεRI in a quiescent state. This mechanism may also explain why low concentrations of allergen given to patients during allergen immunotherapy induce FcεRI nonresponsiveness and therapeutic benefit without inducing systemic anaphylaxis.

Keywords: Mast cells/basophil | IgE | FcεRI | Signal transduction | Desensitization

Article:

Introduction

Mast cells and basophils are the major effector cells in allergic inflammation. When antigen (i.e. pollen) aggregates IgE bound to the high-affinity IgE receptor, FcεRI, on mast cells and basophils, it stimulates signaling cascades that lead to allergic mediator release and allergic disease (hayfever, asthma).

Allergen immunotherapy has proved to be a successful treatment for people with allergic disease, but the mechanism for its efficacy is not known. One hypothesis suggests that allergen immunotherapy simply reduces the amount of allergen-specific IgE available for mast cell/basophil binding. Thus, when patients are subsequently exposed to the allergen, there are not sufficient amounts of specific IgE to sensitize mast cell/basophil FcεRI. In fact, a major anomaly exists as most studies find that immunotherapy does not reduce allergen-specific IgE levels [1,2,3,4,5,6], although mast cell/basophil-induced reactions are diminished [7,8,9]. Specifically, Creticos et al. [1]showed that antigen-specific IgE levels do not decrease following allergy shots; instead antigen-specific IgE levels initially *increase* and do not fall below baseline/initial levels.

Instead, the *intrinsic* IgE-induced reactivity of basophils is reduced following immunotherapy. A recent study typifies this observation where basophils were obtained before and after immunotherapy and challenged with antigen, anti-IgE, or non-IgE-mediated stimuli; extracellular serum concentrations of IgE/IgG were not a factor in these experiments [9]. FcεRI-mediated basophil histamine release was significantly reduced following allergen immunotherapy and no decrease in antigen-specific IgE levels was noted [9]. Therefore, while basophil FcεRI nonresponsiveness is induced during immunotherapy, there is a paradoxical rise in allergen-specific IgE in the serum suggesting that a simple reduction in allergen-specific IgE cannot explain its efficacy.

Like basophils, mast cell nonresponsiveness occurs in patients who have undergone successful allergen immunotherapy where a reduction in the skin prick test, which measures skin mast cell IgE-mediated degranulation in response to antigen challenge, is a strong predictor for clinical efficacy [10]. Similarly, patients allergic to certain drugs (i.e. penicillin) can be 'rush' desensitized by injection of the drug at concentrations that do not trigger an allergic response [11].

While it is clear that mast cells and basophils are capable of being induced to nonresponsiveness by certain in vivo and in vitro scenarios, the mechanisms leading to FcεRI nonresponsiveness are not known. It was hypothesized, based on the observation that the phenotype of IgE nonresponsive mast cells and basophils was similar to naturally occurring, Syk-deficient 'nonreleaser' basophils [12] and lung mast cells [13], that antigen stimulation could induce FcεRI nonresponsiveness and Syk protein reduction. We challenged human skin mast cells with optimal and suboptimal concentrations of antigen and measured mediator release and Syk expression. We show that the induction of IgE nonresponsive skin mast cells and peripheral blood basophils correlates with reduced Syk protein levels. Suboptimal antigen stimulation of freshly purified peripheral blood basophils also resulted in reduced Syk levels. These data suggest that human FcεRI nonresponsiveness is linked to the levels of Syk which can be dramatically reduced in suboptimally challenged cells.

Materials and Methods

Reagents

Monoclonal antiphosphotyrosine (pY) and anti-Lyn Abs were obtained from Santa Cruz Biotechnology, Santa Cruz, Calif., USA. Antiactin was from Sigma, St. Louis, Mo., USA. The anti-Syk, anti-Cbl, and anti-FcεRI-γ Ab were obtained from Upstate, Lake Placid, N.Y., USA. The chimeric human anti-4-hydroxy-3-nitrophenacetyl (NP) IgE Ab and the antigen it detects, NP-BSA, were from Serotec (Oxford, UK) or purified from the cell line JW8 (ATCC, Fairfax, Va., USA). IgE is centrifuged at 60,000 *g* to remove any aggregates. The proteasome inhibitors PSI and MG-132 were from Calbiochem, San Diego, Calif., USA. The antibody 22E7 to the FcεRI-α chain was a generous gift from Dr. J. Kochan.

Tissue Mast Cells

Skin mast cells were purified and cultured as described [14]. Fresh samples of skin or lung were obtained from surgical specimens at the Virginia Commonwealth University Health Systems or through the Cooperative Human Tissue Network of the National Cancer Institute (Columbus, Ohio, USA). All studies were approved by the Human Studies Committee at Virginia Commonwealth University Health Systems. Tissue was cutinto fragments and incubated in a solutioncontaining collagenase, hyaluronidase DNase, and 1% fetal calf serum (digestion buffer) for 2 h at37°C with constant stirring. The dispersed cells were separatedfrom residual tissue by filtration and suspended in HBSS containing 1% FCS and 10 m*M* 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonicacid (HEPES; washing buffer). The remaining tissue was subjectedto two additional digestions as above, and combined with the cellsfrom the first digestion. Cells were resuspended in washing buffer, layered over Histopaque and centrifugedat 700 *g* at room temperature for 20 min.

Enriched skin cells were suspended at 1×10^6 cells/ml in serum-free AIM-V medium (Life Technologies, Rockville, Md., USA) containing 100 ng/ml rhuSCF (Biosource) in 24-well plates (Costar). When the cell number reached approximately 2×10^6 cells/ml, half the cells were split to another well with fresh medium. Cell numbers per sample were adjusted to account for the cells that were split.Total cell number and viability were assessed by trypan blue staining.Cultures were maintained for up to 2 months and were approximately 100% skin mast cells.

Peripheral Blood Basophils

Venous blood (100–300 ml) was collected from healthy, nonmedicated donors who had given informed consent. Basophils were purified as previously described [15, 16].

Measurement of Degranulation and Cytokine Production

All samples were run in duplicate. Mast cells were sensitized with human anti-NP IgE (1–5 μg/ml) for at least 24 h. In some experiments, the proteasome inhibitors PSI or MG-132 (10–100 μ*M*) were added to the cells 30 min prior to activation. Duplicate samples ($1–5 \times 10^5$ cells/condition) were washed and activated in AIM-V media plus 100 μg/ml of soybean trypsin inhibitor with or without NP-BSA. After 30 min, cells were centrifuged and 25 μl of media removed for β-hexosaminidase measurement [17]. Cells were suspended in the remaining media and incubated in a CO_2 incubator for 24–52 h. Cells were centrifuged and the supernatants

stored at –70°C until cytokine measurement. In some experiments IgE-sensitized cells were incubated for 30 h with suboptimal concentrations (0.01–0.1 ng/ml) of NP-BSA, washed and rechallenged as above.

Cytokines were measured using an in-house ELISA. Briefly, granulocyte monocyte-colony-stimulating factor (GM-CSF) or tumor necrosis factor-α (TNF-α) Abs (Pharmingen, San Diego, Calif., USA) were diluted in PBS/1% BSA/Tween (2 μg/ml) and used to coat 384-well plates (Nalge-Nunc, Rochester, N.Y., USA), 4°C for 24 h. The plates were washed 2 times with PBS/Tween and blocked by adding 200 μl blocking buffer (PBS/1% BSA) for 2 h at room temperature. The plates were washed 4 times with PBS/Tween and 100 μl of standards or samples, diluted in PBS/1% BSA/Tween, were added for 24 h at 4°C. Plates were washed 4 times and 100 μl of detection Abs was added for 1 h at room temperature, washed 6 times and 100 μl of a 1:1,000 dilution of Avidin-HRP (Jackson, Bar Harbor, Me., USA) diluted in PBS/1% BSA/Tween was added to each well for 30 min at room temperature. After washing 6 times 100 μl of substrate (100 μl of 3% H_2O_2 to 11 ml 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid diluted 0.3 mg/ml in 0.1 *M* anhydrous citric acid) was added and the OD at 405 nm was measured after 10–20 min.

Basophils were challenged for 30 h in Hanks' balanced salt solution [15] with or without 22E7, anti-IgE, or antigen (Indoor Biotechnologies, Charlottesville, Va., USA). Histamine release was measured as previously described [15].

To examine the rebound in cellular Syk levels and FcεRI responsiveness, NP-IgE-sensitized mast cells ($0.5–10 \times 10^6$ cells/condition) were challenged with or without 0.1 ng/ml of antigen for 24–30 h (to induce desensitization). Cells were washed 3× with AIM-V medium and suspended in AIM-V + SCF for the indicated times. Cells were washed 2× in protein-free medium and subjected to SDS-PAGE and Western blotting as described below. Duplicate samples, treated in parallel, were resensitized with IgE (10 ng/ml) and used to determine FcεRI responsiveness with optimal antigen concentrations.

$[Ca^{2+}]_i$ Measurement using Fura-2 Fluorescence

NP-IgE-sensitized mast cells were adhered to coverslips coated with 0.1% gelatin for 24 h. Cells were loaded with the Ca^{2+}-sensitive probe Fura-2/acetoxymethy (2 μmol/l) for 30 min in a CO_2incubator at 37°C. After loading, cells were washed and incubated in HBSS without cross-linker to obtain a baseline reading followed by the addition of HBSS containing various concentrations of NP-BSA. Intracellular calcium ($[Ca^{2+}]_i$) was measured using an IX-50 inverted fluorescence microscope (Olympus, Tokyo, Japan) in conjunction with a xenon arc excitation source, a photomultiplier, and a photon counter (Ionoptix, Milton, Mass., USA). Data were acquired and analyzed with Ionwizard software provided by the manufacturer. Epifluorescence was measured from ≥5 mast cells, and the values in $[Ca^{2+}]_i$ determined as the fluorescence ratio *(R)* ofCa^{2+}-bound Fura-2 (340 nm) to unbound Fura-2 (380 nm), withemission collected at 510 nm. *R* was converted to free Ca^{2+} concentration using an in vitro standard curve generated with a calibration kit (Molecular Probes, Eugene, Oreg., USA).

Small Inhibitory RNA-Mediated Inhibition of Syk Expression

Four pooled SMARTselected human Syk small inhibitory RNA (siRNA) duplexes with UU overhangs and a 5′-phosphate on the antisense strand were obtained from Dharmacon Research, Lafayette, Colo., USA. As a control, four nonspecific siRNAs with equal GC content were used. The siRNA duplex was introduced into human skin mast cells using a liposome transfection approach. Liposome/siRNA complexeswere formed at room temperature using 5 µl of 20 µ*M* siRNA, 3 µl of LipofectAMINE^{TM} 2000 (Invitrogen), and 100 µl of serum-free DMEM culturemedium (Invitrogen). The resulting solution was added dropwise to each 24-well culture dish containing ~1×10^5 MCs. The cells were incubated 4 h at 37°C. One milliliter of serum-enriched medium was then added, and the cells were culturedfor an additional 30 h. The transiently transfected cells were harvested, and IgE-mediated degranulation and cytokine production measured after IgE sensitization. In parallel, the levels of Syk and actin protein were measured using the below SDS-PAGE immunoblot approach.

Western Blotting

Mast cells were lysed in denaturing lysis buffer at $0.5–10 \times 10^7$ cells/ml. The protocol for this is described by Tkaczyk et al. [18]. Expression of Syk, Lyn, and actin protein was measured by Western blotting as previously described [12, 15].

Band intensities were quantitated using a molecular imaging package from Biorad (The Discovery Series, Quantity One, Quantitative Software). In some experiments band intensities are presented as a ratio of Syk to actin to compensate for gel loading and blot stripping/reprobing variations. Degranulation was measured in each Western blotting/immunoprecipitation experiment by measuring β-hexosaminidase as described above.

Results

FcεRI Nonresponsiveness due to Long-Term Antigen Stimulation

The mechanism for FcεRI nonresponsiveness after long-term antigen stimulation is not clear and has not been demonstrated with human mast cells. We tested human skin mast cells for their ability to become unresponsive to IgE stimuli following long-term antigen stimulation. NP IgE-sensitized cells were challenged with or without optimal (10 ng/ml) or suboptimal (0.1 ng/ml) concentrations of NP-BSA up to 52 h. Supernatants were collected after this initial desensitization step and examined for degranulation (prerelease). Cells were resensitized with IgE for 2 h, washed, and rechallenged with optimal concentrations of NP-BSA for 30 min and β-hexosaminidase measured in the supernatants. As expected, no desensitization was observed in cells not exposed to cross-linker (fig. 1a) and optimal concentrations of antigen induced robust degranulation (fig. 1c). As shown in figure 1b, FcεRI-mediated degranulation was optimally inhibited when cells were first exposed to suboptimal concentrations of antigen for 24 h. This nonresponsiveness was not simply due to the loss of IgE as FACs analysis with FITC-labeled anti-IgE Abs revealed the presence of IgE for up to 3 days postsensitization (fig. 1d). Compounds which bypass FcεRI (A23187) induced a robust response indicating a specific defect in the FcεRI signal transduction pathway, reminiscent of nonreleaser basophils.

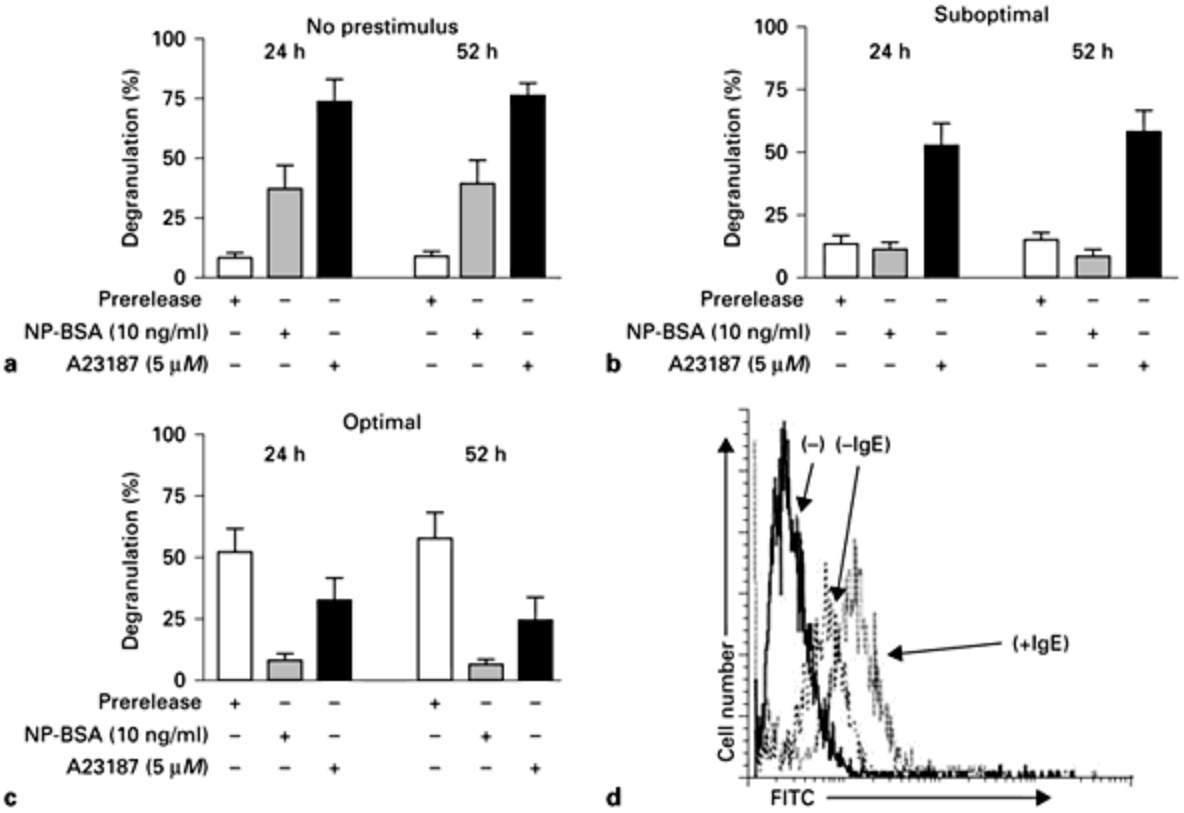

Fig. 1. In vitro desensitization of FcεRI on human skin mast cells. Skin mast cells (2×10^5 cell/well) were sensitized with NP-IgE (1 μg/ml), washed and challenged for 24 or 52 h with no cross-linker (**a**), suboptimal (0.1 ng/ml; **b**) or optimal (10 ng/ml; **c**) concentrations of NP-BSA. β-Hexosaminidase was measured in the supernatants (prerelease). Cells were resensitized with IgE and challenged with optimal concentrations of NP-BSA (10 ng/ml) or A23187 (5 μ*M*) for 30 min. All experiments were performed in duplicate. The results show the standard error of the mean from 3 separate experiments. **d** Skin mast cells were incubated with (+) or without (–) IgE and desensitized with suboptimal concentrations of NP-BSA for 52 h (as in **b**). Cells were washed and resensitized (+IgE) or not (–IgE) with NP-IgE. They were washed and incubated with FITC-labeled anti-IgE and analyzed by FACs.

Previous studies showed decreased levels of Syk protein were associated with human basophil FcεRI nonresponsiveness [12, 19] and human mast cells may have a similar nonreleaser phenotype [13]. Thus, it was hypothesized that nonresponsive skin mast cell FcεRI induced by suboptimal antigen stimulation was due to decreased Syk levels. As shown in figure 2a, NP-IgE-sensitized skin mast cells begin to lose Syk expression at antigen concentrations which did not induce detectable degranulation and 10,000 times less (0.001 ng/ml) than that required for optimal degranulation (10 ng/ml). Syk was decreased in cells challenged from 0.0001 to 10 ng/ml of antigen for 24 h, while Lyn levels remained unchanged. High doses of antigen (>100 ng/ml), which causes excessive receptor cross-linking and reduced mediator release, did not reduce Syk levels. No changes were noted in FcεRI-γ protein levels under the same conditions (data not shown). Challenge with suboptimal concentrations of antigen reduce Syk levels at approximately 24 h; shorter incubation times did not reduce Syk levels (fig. 2b). Similar results were observed with highly purified human lung mast cells (not shown).

Peripheral blood basophils are similar to tissue mast cells as both cell types express high amounts of FcεRI which mediates IgE degranulation. Freshly purified basophils were challenged

with or without FcεRI stimulation for 24–30 h and Syk expression examined by Western blot. As seen in figure 2c, basophil Syk is reduced in response to IgE-mediated stimulation. As with mast cells, Syk reduction occurred at antigen concentrations that did not induce noticeable degranulation (0.01 ng/ml). These results suggest that suboptimal FcεRI cross-linking induces basophil nonresponsiveness through a mechanism which reduces Syk levels. In addition, reduced Syk levels due to low concentrations of IgE receptor cross-linker may be a common mechanism regulating human FcεRI cellular responsiveness.

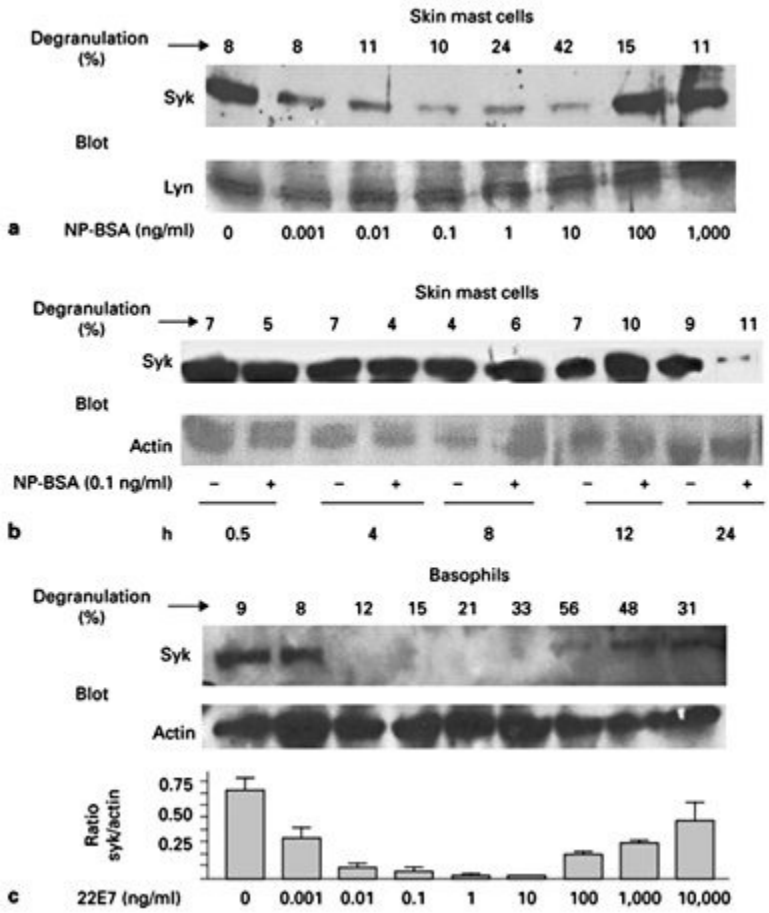

Fig. 2. FcεRI desensitization on human skin mast cells correlates with reduced levels of Syk. NP-IgE-sensitized skin mast cells ($1.8–2.0 \times 10^6$ cell equivalents/lane) were challenged for >24 h with or without increasing concentrations of cross-linker (**a**) or with or without 0.1 ng/ml of cross-linker for various times (**b**). Cells were washed, lysed, and separated by SDS-PAGE and Western blotted with antibodies against Syk, Lyn or actin as described in Methods. Supernatants from parallel-treated cells were examined for degranulation. Results are representative of 2 separate experiments. **c** Peripheral blood basophils (98% purity) were challenged for 30 h with varying concentrations of anti-IgE receptor antibodies (22E7) and Syk levels monitored with Western blotting (3.2×10^5 cell equivalents/lane). The error bars in the densitometric analysis represent results from 3 separate experiments.

Reduction in Syk Levels and Cytokine Release from Human Skin Mast Cells

We next determined if Syk reduction occurred at antigen concentrations which induce mast cell cytokine production. As seen in figure 3, densitometric analysis demonstrated reduced Syk levels at antigen concentrations that did not induce degranulation or the production and secretion of TNF-α and GM-CSF. Similar results were obtained with IL-5 cytokine production (data not

shown). Thus, suboptimal doses of antigen induce Syk reduction at concentrations 1,000 times less than that required for cytokine production.

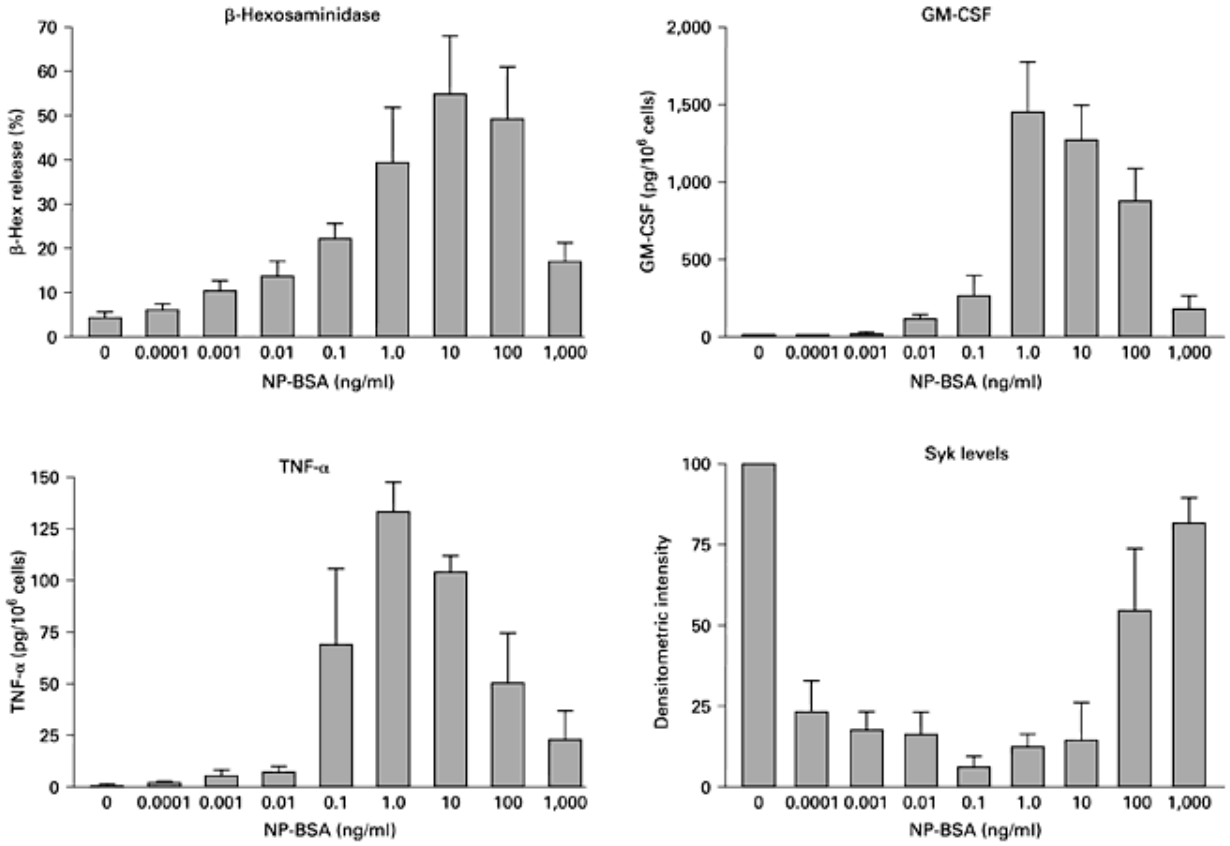

Fig. 3. FcεRI-induced Syk reduction occurs at antigen concentrations that do not induce degranulation or cytokine production. NP-IgE-sensitized skin mast cells were challenged for 30 min (β-hexosaminidase, β-Hex) or 24 h (GM-CSF, TNF-α or Syk levels) with or without increasing concentrations of cross-linker. Supernatants were collected and assayed for mediator release. Cells were washed, lysed, and separated by SDS-PAGE and Western blotted with antibodies to and Syk and bands visualized with ECL (not shown). The Syk blots were scanned and band intensities quantitated using densitometric analysis. Results show the standard error of the mean from 4 (degranulation) or 3 (cytokine release) experiments.

Syk Is Reduced with Suboptimal Antigen Stimulation through a Calcium-Independent, Proteasome-Dependent Mechanism

The mechanism leading to antigen-induced Syk deficiency was further explored. Reduced Syk levels with suboptimal antigen concentrations (0.001–0.1 ng/ml) occurred at concentrations that were independent of the release of $[Ca^{2+}]_i$ store release (fig. 4a). Monitoring $[Ca^{2+}]_i$ in response to suboptimal concentrations inducing Syk deficiency (0.001 and 0.01 ng/ml) for longer periods of time (up to 6 h) failed to detect store release (not shown). As seen in figure 4b, skin mast cells incubated with suboptimal antigen had low levels of Syk. However, Syk levels and IgE-mediated degranulation were increased under these conditions if the proteasome inhibitors PSI or MG-132 were added to the cells before FcεRI challenge. We also observed similar Syk mRNA levels in cells challenged with suboptimal antigen concentrations and nonchallenged cells (data not shown). These results suggest that mast cell challenge with suboptimal antigen concentrations

induces a reduction in Syk protein through a posttranslational, proteasome-dependent mechanism.

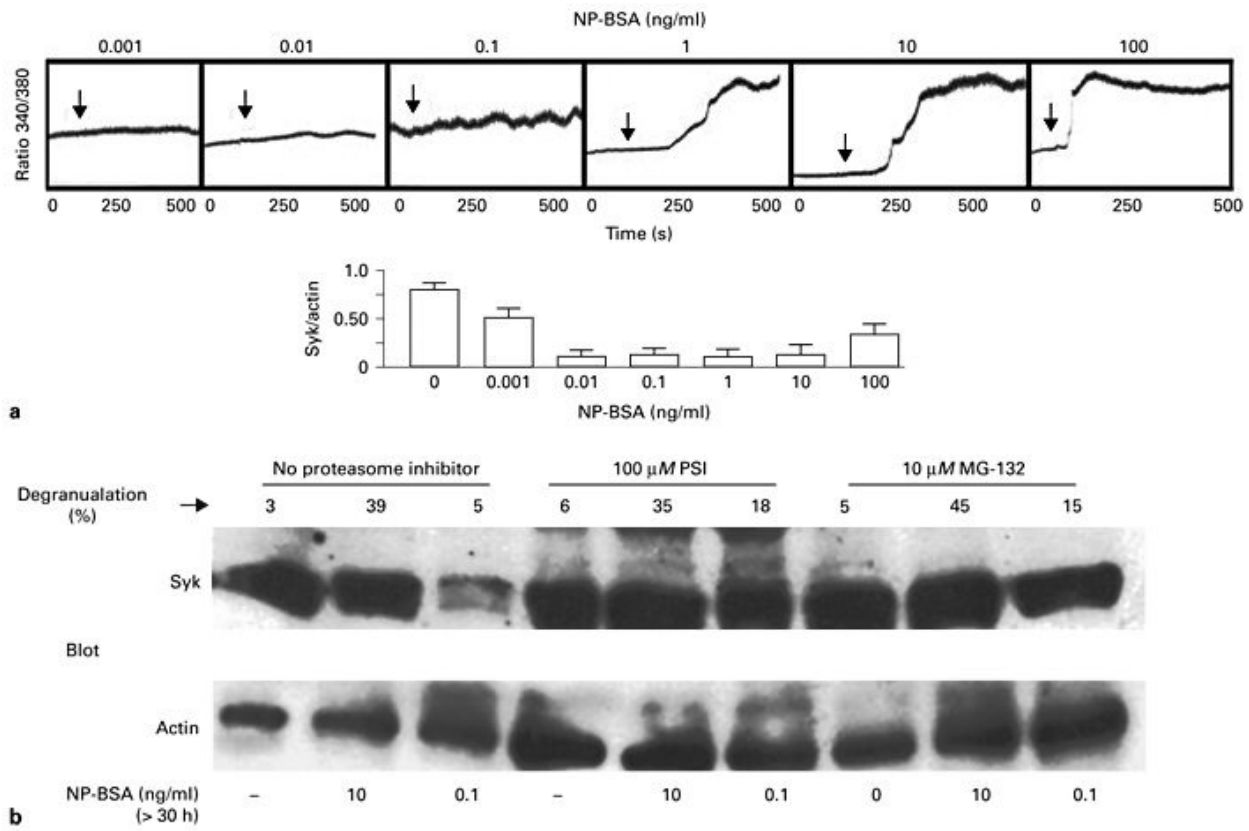

Fig. 4. a Syk reduction with suboptimal antigen concentrations is calcium independent. Fura-2-labelled mast cells were challenged with the indicated concentrations of NP-BSA (arrows) and the $[Ca^{2+}]_i$ measured for 500 s, indicated on the y-axis, as described in Methods. In parallel, similarly treated mast cells were Western blotted sequentially with Syk and actin Abs and the band intensities presented as a ratio as described above. **b** Proteasome inhibitors block FcεRI-induced Syk reduction. NP-IgE-sensitized mast cells (1.2×10^6 cell equivalents/lane) were challenged for 24 h with or without 0.1 ng/ml of cross-linker. Some cells were treated with the proteasome inhibitor PSI or MG-132 (100 μ*M*) for 30 min prior to challenge. Cells were washed, lysed, and separated by SDS-PAGE and Western blotted sequentially with antibodies against Syk and actin as described in Methods. Supernatants from parallel-treated cells were examined for degranulation after 38-hour challenge with NP-BSA and expressed as the percent release of β-hexosaminidase. Results are representative of 2 separate experiments.

A Rebound in Cellular Syk Correlates with FcεRI Responsiveness

Based on the observation that antigen-induced FcεRI nonresponsiveness correlated with reduced Syk levels, we investigated if a rebound in Syk levels correlated with the loss of FcεRI nonresponsiveness. IgE-sensitized mast cells were challenged with suboptimal antigen concentrations as above. Cells were washed free of antigen. IgE-mediated degranulation and Syk levels were monitored at different time points. As expected, Syk levels were dramatically lower in cells challenged for 30 h with suboptimal antigen and the cells failed to respond to FcεRI stimuli (fig. 5a). Cells began to respond to antigen between 12 and 24 h postantigen challenge; cells examined at shorter time points remained IgE nonresponsive and had reduced Syk levels. Increased Syk levels were observed at approximately 18 h after removal of antigen and paralleled the loss of FcεRI nonresponsiveness(fig. 5a).

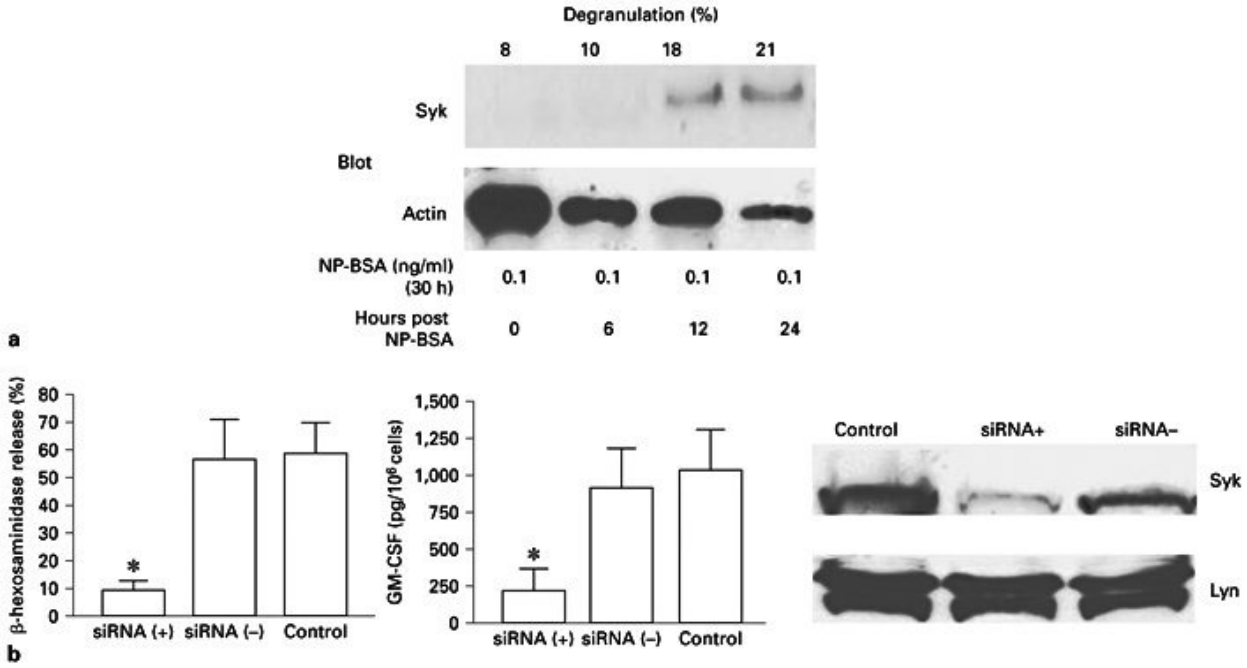

Fig. 5. a Increased Syk levels parallel the loss of FcεRI desensitization. NP-IgE-sensitized mast cells were challenged with or without 0.1 ng/ml of NP-BSA for 30 h. Cells were washed and incubated in medium containing NP-IgE (10 µg/ml). Degranulation (5×10^5 total cells) and Syk levels (2.2×10^6 cells/time point) were monitored at 0, 6, 12 and 24 h after the removal of antigen. For degranulation, cells were challenged with or without optimal concentrations of antigen (in duplicate as in fig. 1) and the percent β-hexosaminidase release calculated in the supernatant. In parallel, Syk levels were measured by Western blot. Results are representative of 3 similar experiments. **b** Reduction in Syk protein correlates with reduced IgE-mediated degranulation and cytokine production. Mast cells (2.0×10^5 cells/ml) were transfected with Syk siRNA (siRNA+), scrambled controls (siRNA–), or no siRNA (control). Each group was examined for mediator release by sensitizing with NP-IgE (10 µg/ml) for 2 h and challenging with antigen (left panel) or examined for Syk levels by SDS-PAGE and Western blotting as described (right panel). The asterisk indicates a significant decrease in degranulation ($p = 0.024$) and cytokine production ($p = 0.03$) using the Student t test in conditions pooled from 3 different experiments.

To further determine a cause and effect relationship between reduced Syk levels and inhibition of mast cell mediator release we used siRNA to evaluate the consequences of decreased Syk expression in human skin mast cells. As seen in figure 5b, inhibition of Syk expression in skin mast cells resultedin an inhibition of IgE-mediated degranulation and cytokine production. The siRNA treatment did not affect total β-hexosaminidase levels inside the cell (data not shown). These data further support a critical role of Syk in human mast cell signal transduction. In addition, these are the first data demonstrating the ability of skin mast cells to be transfected with siRNA duplexes.

Discussion

We previously showed that nonreleaser basophils, which also demonstrate FcεRI nonresponsiveness, had reduced Syk levels [12, 19]. We hypothesized that low concentrations of antigen could induce FcεRI nonresponsiveness through a reduction in Syk protein. In the studies presented here we demonstrate that human skin mast cell FcεRI becomes nonresponsive with antigen concentrations that do not induce significant mediator release. This nonresponsiveness

correlates with reduced levels of Syk, similar to nonreleaser basophils. Low concentrations of antigen also lead to reduced Syk levels in peripheral blood basophils. Syk appears to be downregulated through proteasome degradation as agents that block these events also block Syk reduction. The initial steps leading to Syk reduction appear to be calcium independent. When antigen is removed the mast cells become responsive to IgE-mediated stimuli after 24 h in culture. The loss of FcεRI nonresponsiveness paralleled increases in Syk levels. The observation that reduced Syk levels occurs at the protein level is similar to those observed in nonreleaser basophils [19,20,21].

Our data demonstrates that the loss of Syk occurs only after prolonged incubation with antigen. This is very different from what was reported in the original description of 'desensitization' of basophils [22,23,24]. In these studies desensitization occurred within minutes when the cells were challenged with antigen in the absence of calcium. This acute mechanism for FcεRI downregulation does not appear to involve FcεRI/antigen internalization or Syk loss. Recent studies by MacGlashan [25]demonstrated that antigen stimulation of basophils induces downregulation operating before or at the level of Syk phosphorylation. In these experiments basophils were challenged with antigen for less than 2 h before challenge with anti-IgE. The levels of Syk phosphorylation were transient with antigen stimulation and no reduction in Syk levels was noted, probably due to the reduced prechallenge time.

Recent studies by this group found that optimal and suboptimal concentrations of antigen led to reduced Syk expression similar to what was observed in the present study (fig. 2c) [21]. The studies presented here confirm the studies in basophils and extend these observations in skin mast cells. Taken together, these results suggest reductions in Syk levels through antigen stimulation may act to keep these inflammatory cells in a quiescent state.

We also observed that Syk levels were lower in mast cells/basophils that were optimally challenged with antigen but had maximal mediator release. This is not surprising in that degranulation occurs within 30 min following activation while cytokine production occurs after 24 h. Therefore, there are likely adequate amounts of Syk protein to mediate the signaling cascade leading to mediator release.

We observed that antigen-induced Syk reduction was mediated through ubiquitination and proteasomal degradation. Rodent mast cell-like cells (RBL-2H3) and human basophils demonstrated Syk ubiquitination after FcεRI engagement with optimal concentrations of antigen and suggest a similar molecular mechanism for downregulation of receptor complexes by targeting ubiquitinated Syk to the proteasome for degradation [21, 26]. The mechanisms presented here and previously may explain how a 'resting' cell remains nonactivated after suboptimal antigen challenge while Syk reduction with optimal concentrations of antigen is consistent with a mechanism for 'turning off' ongoing mast cell mediator responses.

The precedence for selective protein degradation through activation of immune receptors is strong. Ubiquitination is a posttranslational modification that controls the fate of proteins through selective proteasome degradation. In antigen-stimulated T cells, ZAP-70 (a kinase similar to Syk) is rapidly degraded via a calpain-dependent mechanism [27]. Paolini and Kinet [28] found FcεRI engagement on RBL-2H3 resulted in Syk ubiquitination and Syk kinase

activity is required for its own ubiquitination [29]. Similarly, B cell receptor stimulation induces Syk ubiquitination [30, 31]. Others have shown that both Syk and ZAP-70 become ubiquitinated in response to antigen stimulation in other cell types [32]. Receptor-mediated activation of other kinases similarly 'tags' them for ubiquitination-dependent degradation [26, 33, 34].

It is possible that low concentrations of antigen induces IgE nonresponsiveness through a Syk-independent mechanism. However, we showed that reducing the level of Syk with siRNA correlated with reduced IgE signaling leading to mediator release (fig. 5b). This was expected as we and others have demonstrated that FcεRI-induced mediator release is dependent on the levels of Syk protein [12, 20, 35]. Reduction in levels of other molecules leading to a lack of IgE-mediated degranulation have been reported in rodent cells [36, 37], while Lyn deficiency appears to have little affect [38]. Nonetheless, adequate quantities of Syk are clearly required for a full response through FcεRI on human mast cells.

The implications for these studies are far-reaching. No hypothesis has been able to explain how graded increases in allergen dosing (up to 15–20 μg of major allergen) given during immunotherapy can be injected without inducing anaphylaxis. It has been assumed that as the dose of allergen is slowly titrated up, some degree of 'tolerance' is induced which renders the next dose safe. It is plausible that the mechanisms underlying chronic allergen stimulation and concomitant FcεRI nonresponsiveness may involve decreased Syk levels resembling nonreleaser mast cells/basophils. How could chronic, low-dose antigen stimulation induce degradation of FcεRI-specific kinases without affecting protein levels in other cells (as with nonreleaser basophils and possibly mast cells)? The following hypothesis is proposed (fig. 6).

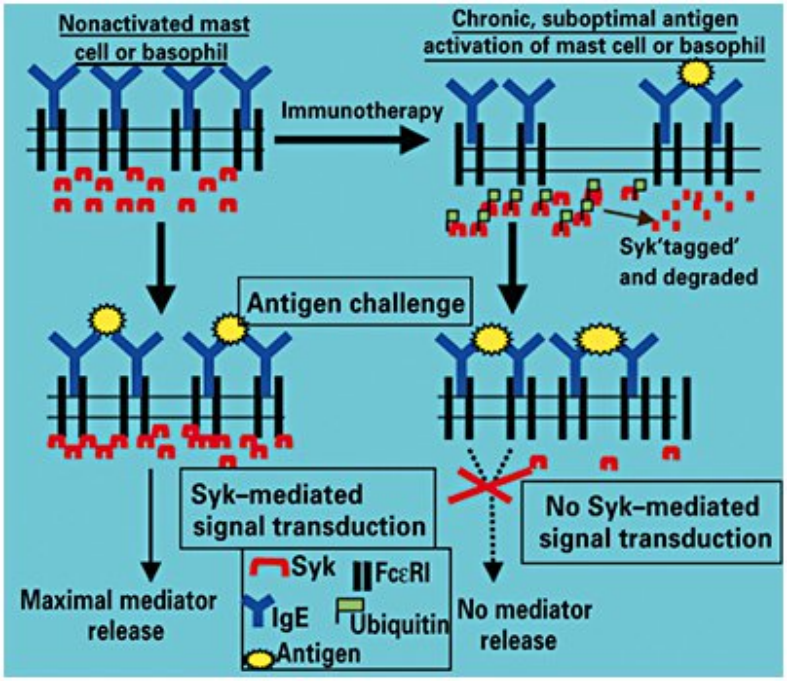

Fig. 6. Chronic antigenic stimulation results in lower Syk kinase levels. Normally, when atopic donors are challenged with antigen there is degranulation mediated through Syk. However, low doses of antigen challenge, through allergen immunotherapy, suboptimally cross-link surface-bound IgE on mast cells and basophils resulting in the proteasome degradation of Syk. When cells are then antigen challenged there is less Syk signaling and thus inhibited mediator release.

Allergen immunotherapy, which aims to induce FcεRI nonresponsiveness with suboptimal doses of antigen, initially induces antigen-specific IgE and is given at a dose that does not result in noticeable symptoms. Fatalities have been associated with allergen skin testing and immunotherapy; thus ensuring the patient receives a dose of allergen that will induce desensitization – but not anaphylaxis – is an important safety concern. Low IgE-FcεRI cross-linking leads to the downregulation and degradation of FcεRI receptor-associated kinases through ubiquitination and proteasome degradation. The recurrent stimulation (induction involves weekly shots) with increasing doses of allergen would ensure continued, subacute activation and kinase ubiquitination, while the specific, high-affinity interaction between mast cell/basophil surface IgE with antigen would ensure only downregulation of kinase levels in these cells. In preliminary experiments we found that donors undergoing specific allergy vaccination have reduced basophil histamine release (as others have found [9]) and undetectable levels of Syk during the initial up-dosing period (Kepley, unpubl. data). The data presented here and previously [21] provide mechanistic evidence to support the above hypothesis.

In conclusion, we show that proteasome-mediated degradation of FcεRI-associated Syk with low concentrations of antigen, for extended periods of time, leads to FcεRI nonresponsiveness on mast cells. We also confirm previous studies in human basophils by demonstrating that antigen challenge induces reductions in Syk protein levels. The resulting reduction in kinase levels would blunt the signaling process and inhibit IgE cell responsiveness to antigenic stimulation. This mechanism may account for the clinical success observed during the initial, ‘up-dosing’ that occurs with allergen immunotherapy.

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