



Insulin potentiates FcεRI-mediated signaling in mouse bone marrow-derived mast cells

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ABSTRACT

Factors contained in physiological microenvironments in tissues where mast cells differentiate and reside may influence mast cell responsiveness and modify antigen-dependent activation. A possible direct or indirect role of mast cell responses in diabetes mellitus prompted us to study the impact of insulin treatment on antigen triggered signaling pathways downstream of FcεRI in bone marrow-derived mouse mast cells (BMMCs). We found that insulin alone stimulates tyrosine phosphorylation of tyrosine kinases Lyn, Syk, Fyn, the adapter protein Gab2 (Grb2-associated binding protein 2), Akt and activates ERK, JNK and p38 kinase. Effect of insulin on FcεRI signaling pathways was marked by enhanced phosphorylation of Lyn, Fyn, Gab2 and Akt. Furthermore, BMMC stimulated with antigen in the presence of insulin responded with enhanced protein kinase θ (PKCθ) activity and increased JNK phosphorylation when compared to BMMC triggered with antigen alone. Functional studies reveal enhanced degranulation and altered cytoskeletal rearrangement when BMMCs were treated simultaneously with insulin and antigen. Our results suggest that insulin tunes antigen-mediated responses of mast cells.

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1. Introduction

Mast cell activation leads to the secretion of preformed granule-associated mediators, newly generated lipid mediators and a wide variety of cytokines and chemokines. Signaling studies of the biochemical events leading to mast cell responses have been mainly focused on the molecular pathways downstream of FcεRI (Kinet, 1999). Upon FcεRI aggregation, immunoreceptor tyrosine-based activation motifs of the γ and β subunits become phosphorylated by the Src family tyrosine kinase Lyn (Penhallow et al., 1995) and recruit the protein tyrosine kinase Syk (Costello et al., 1996), which in turn phosphorylates LAT (linker for activation of T cells) (Saitoh et al., 2000) which functions as scaffold to organize other signaling proteins such as SLP-76 (SH2-domain-containing leukocyte protein of 76 kDa) (Hendricks-Taylor et al., 1997), phospholipase C-γ (Musch and Siegel, 1986) and Vav (Margolis et al., 1992). Concomitant activation of Fyn kinase initiates signaling through a complementary but distinct pathway leading to PI3K activation mediated by Gab2 (Parravicini et al., 2002). The synergic activa-

tion of these two pathways orchestrates antigen triggered mast cell responses.

Mast cell responses are not limited to stimulation through IgE and antigen-mediated crosslinking of FcεRI (Gilfillan and Tkaczyk, 2006). They express many more receptors for endogenous mediators that upon ligand binding elicit the release of mediators from mast cells, with c-Kit one of the best characterized. c-Kit stimulation is essential for growth, survival and differentiation of mast cells (Tsai et al., 1991) but it also potentiates FcεRI-mediated signaling resulting in enhanced degranulation and cytokine production (Vosseller et al., 1997). Other receptors that have been described to modulate antigen-dependent mast cell activation, include G-protein-coupled receptors for adenosine (Tilley et al., 2000), complement component 3a (Erdei et al., 1995), sphingosine 1-phosphate (S1P) (Jolly et al., 2004) and toll-like receptors (Fehrenbach et al., 2007; Qiao et al., 2006). In order to understand physiological mast cell responses, it is of importance to study the influence of such ligands present in the microenvironment where mast cells mature and dwell. Mast cells also reside in insulin responsive tissues and recent reports indicate that insulin might have a direct impact on mast cell function (Geoffrey et al., 2006). Signaling downstream of the insulin receptor is best characterized in the context of vesicle associated glucose transporter 4 (GLUT4) translocation to the cell membrane and subsequent glucose uptake (Gould and Holman, 1993). In mast cells, insulin promotes survival in a PI3K-dependent manner and enhances IgE-dependent degranulation (Lessmann et al., 2006). Thus, activation of PI3K is an event

Abbreviations: BMMC, bone marrow-derived mouse mast cell; PKC, protein kinase C; Gab2, Grb2-associated binding protein 2; GLUT4, glucose transporter 4; HSA, human serum albumine; WIP, Wiskott–Aldrich syndrome protein-interacting protein.

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shared by many receptors known to modulate mast cell responses (Kim et al., 2008).

Here we report insights to the molecular mechanisms through which insulin may modulate FcεRI-mediated signaling. We found that insulin ligation is able to trigger the phosphorylation of Lyn, Syk and Fyn, PI3K as well as the MAP kinases JNK, ERK and p38. We provide evidence that insulin-mediated signaling events integrate at several levels with the signaling pathways initiated by FcεRI aggregation. These biochemical events suggest that insulin may modulate and amplify mast cell activity and thereby tune antigen-mediated mast cell responses.

2. Materials and methods

2.1. Cells and cell culture

Bone marrow cells were obtained by flushing the femur and tibia bones of C57BL/6 mice and were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 1% X63Ag8-653- conditioned medium as source of IL-3 (Karasuyama and Melchers, 1988), 2 mM L-glutamine, 0.1 M HEPES, 50 units/ml penicillin, 50 µg/ml streptomycin and 0.1 mM non-essential amino acids. Cells were passed twice weekly at a concentration of 1×10^6 cells/ml in fresh medium. After 4–6 weeks of culture, >90% of cells were mast cells as assessed for IgE binding by FACS analysis. BMMCs were incubated with anti-DNP mouse IgE (clone SPE-7; Sigma–Aldrich) and anti-IgE-PE (BD Biosciences). Cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences).

2.2. β-Hexosaminidase release assay

10^6 BMMCs were incubated with anti-DNP IgE ($0.2 \mu\text{g}/10^6$ cells) in Tyrode's buffer (10 mM HEPES pH 7.4, 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 0.1% BSA; Sigma–Aldrich) for 1 h at 4 °C on a rotating wheel. After washing, cells were resuspended in Tyrode's buffer, placed in a 37 °C water bath for 10–15 min and thereafter degranulation was initiated by addition of DNP–human serum albumine (DNP–HSA; Sigma–Aldrich), insulin (Sigma–Aldrich) or a mixture of both. After 20 min, cells were centrifuged and pellets were resuspended to their original volume with Tyrode's buffer containing 0.5% Triton X-100 (Bio-Rad). Aliquots of supernatants and cell lysates were incubated in duplicates with substrate solution (1.3 mg/ml p-nitrophenyl-β-D-2-acetamido-2-deoxyglucopyranoside in 0.1 M citrate, pH 4.5; Sigma–Aldrich). Percentage of degranulation was calculated by the formula $[S/(S+P)] \times 100$, where S and P are the β-hexosaminidase contents of the supernatant (S) and pellet (P) from each sample. OD for buffer alone was subtracted from S and P.

2.3. Western blotting and immunoprecipitation

For immunoprecipitation experiments, BMMCs were preloaded with IgE for 1 h as described above and then stimulated for the indicated time after an initial resting period of 15 min at 37 °C with DNP–HSA, insulin (Sigma–Aldrich) or a mixture of both at the concentrations indicated. After centrifugation, cells were lysed for 30 min in ice cold lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 3 mM diisopropyl fluorophosphate, 1 mM PMSF, 0.5 mM Na₃VO₄ and 5 mM NaF; Sigma–Aldrich, Complete™ protease inhibitor cocktail; Roche Applied Science) for 30 min on ice. Postnuclear supernatants were either directly separated by SDS-PAGE and subjected to Western blot analysis for phospho-Akt, phospho-ERK, phospho-p38, p38, phospho-JNK and JNK (all from Cell Signaling Technology) or they were subjected to immunoprecipitation with antibodies to Syk, Lyn, Fyn (Santa Cruz), PKCθ (BD Biosciences), Gab2 (Millipore) and protein G-sepharose

beads. After three washing steps in lysis buffer and one with lysis buffer without detergent, immunoprecipitates were separated by SDS-PAGE and analyzed by Western blotting by probing with anti-phosphotyrosine 4G10 (Millipore).

2.4. PKCθ kinase assay

BMMCs were loaded with IgE, stimulated and lysed (lysis buffer supplemented with 0.8 mM sodium pyrophosphate and 10 mM β-glycerophosphate) as described above and PKCθ was immunoprecipitated from lysates. Immunoprecipitates were washed twice with lysis buffer and twice with kinase buffer (20 mM PIPES, 10 mM MgCl₂, 0.1 mM EGTA, pH 7.4). After last wash, beads were resuspended in 20 µl kinase buffer supplemented with ATP (0.1 mM) and myelin basic protein (dephosphorylated MBP, 20 µg/sample; Millipore) as substrate. The mixture was incubated for 20 min at 30 °C and then reducing sample buffer was added to stop the reaction. Kinase and substrate were separated by SDS-PAGE and Western blotting for PKCθ and phospho-MBP (Millipore) was performed.

2.5. Determination of cellular F-actin

10^6 BMMCs incubated with IgE in Tyrode's buffer as described above were rested for 15 min at 37 °C and then stimulated with DNP–HSA diluted to 100 ng/ml, insulin (100 nM) or a mixture of both in Tyrode's buffer for the indicated time. Cells were immediately fixed for 10 min by adding 4% formaldehyde (2% final concentration). They were then permeabilized and stained in a single step with 0.1% Triton X-100 and 1 µg/ml phalloidin-TRITC (Molecular Probes) in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl₂, pH 6.9; Sigma–Aldrich) for 40 min and analyzed by FACS.

2.6. Statistical analysis and densitometry

Statistical significance of differences between experimental groups was determined with a two-tailed Student's *t*-test for paired data. Blots were scanned and analyzed with ImageJ software (<http://rsb.info.nih.gov/ij>). Only non-saturated signals were considered.

3. Results

3.1. Insulin enhances IgE-mediated degranulation of bone marrow-derived mast cells

In a rat model of diabetes a significant reduction in the number of degranulated mast cells and in histamine release has been observed in bronchial segments (Cavalher-Machado et al., 2004). Moreover, insulin signaling leads to vesicle trafficking, namely the transport of GLUT4 containing vesicles to the membrane (Tsakiridis et al., 1994). We then tested the hypothesis whether insulin might have an effect on the release of preformed mediators stored in the granules of mast cells. BMMCs were stimulated with antigen, insulin or antigen together with insulin at indicated concentrations and the release of the granular enzyme β-hexosaminidase was determined (Fig. 1A). Incubation of mast cells with insulin did not result in measurable degranulation as no β-hexosaminidase enzymatic activity over background was observed (Fig. 1 A and B). On the other hand, treatment of mast cells with insulin and simultaneous crosslinking of FcεRI resulted in increased degranulation (>20%) compared to stimulation with antigen alone. Thus, insulin receptor generated signals seem to feed into signal transduction pathways downstream of FcεRI. The dose of 100 nM insulin was chosen after we

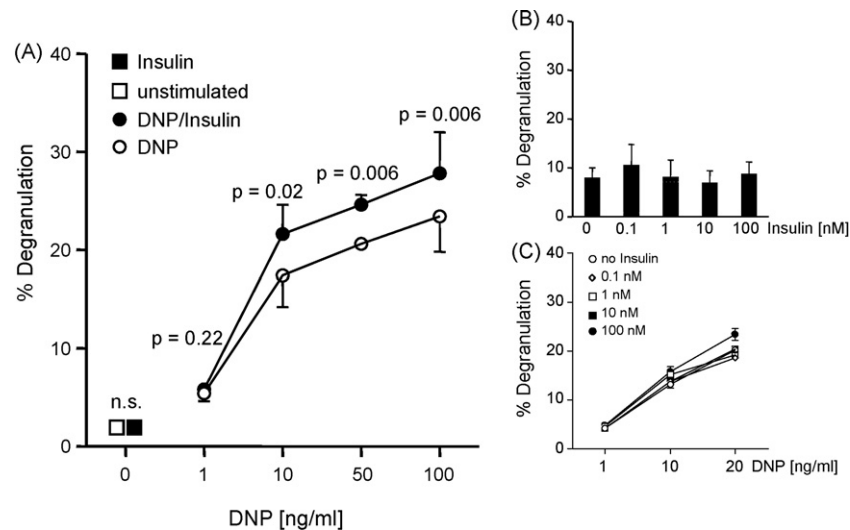


Fig. 1. Insulin augments IgE-mediated in vitro degranulation of BMMCs. (A) IgE preloaded BMMCs were left unstimulated, stimulated either with insulin (100 nM), DNP–HSA or simultaneously with insulin and DNP–HSA at indicated concentrations for 20 min. Degranulation was determined by β -hexosaminidase assay. Results are the mean $\pm$ SD of four experiments done in duplicates from two independently derived cell lines. Statistical analysis was performed using the Student's *t* test. (B) BMMCs were incubated with various concentrations of insulin. Results are the mean $\pm$ SD of two experiments. (C) IgE preloaded BMMCs were stimulated simultaneously with insulin and DNP–HSA at indicated concentrations for 20 min. Results are the mean $\pm$ SD of two experiments.

found that in preliminary experiments antigen-mediated degranulation was consistently (over a range of antigen concentration) augmented at this concentration (Fig. 1A and C). Some augmentation was also observed for a physiological concentration of 1 nM insulin but only at one concentration of antigen (10 ng/ml). We then performed experiments to study the biochemical events underlying the observed enhanced degranulation of BMMCs in response to Fc ϵ RI ligation in the presence of insulin.

3.2. Early signaling events after BMMC stimulation with antigen

Two distinct pathways have been described whose synergic activation cumulates in degranulation. The first pathway involves activation of protein tyrosine kinases Lyn and Syk while in the second pathway protein tyrosine kinase Fyn and the adapter protein Gab2 are activated (Parravicini et al., 2002). Interestingly all of these molecules were phosphorylated upon stimulation of BMMCs with

insulin alone (Fig. 2). In the case of Syk and Fyn up to the same level as when cells were stimulated with antigen alone. A suboptimal dose of antigen in respect to the degranulation response was chosen as the signal transduced through Fc ϵ RI at higher antigen concentrations was too strong to detect the contribution of the insulin stimulated pathways. Along the first pathway, the signal for Lyn phosphorylation (Fig. 2A) detected upon combined stimulation of BMMCs with antigen and with insulin was stronger than in cells stimulated with antigen alone but enhanced Lyn phosphorylation was not accompanied by increased Syk phosphorylation (Fig. 2B) when BMMCs were stimulated with insulin and antigen simultaneously. Along the second pathway Fyn phosphorylation (Fig. 2C) was only modestly increased upon combined stimulation with antigen and insulin. The signal observed for Gab2 phosphorylation (Fig. 2D) instead was clearly amplified when BMMCs were stimulated with antigen and insulin together. These results indicate that Lyn and Gab2, a member of the insulin receptor substrate

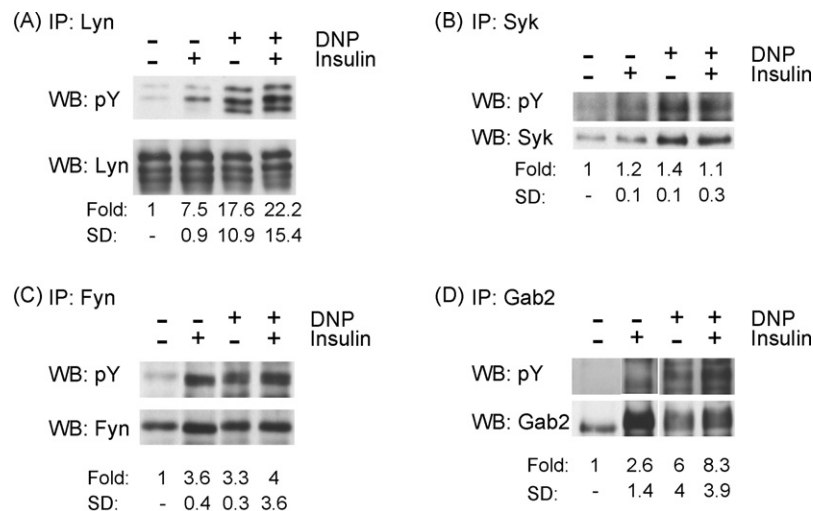


Fig. 2. Insulin activates Lyn/Syk as well as Fyn/Gab2 signaling pathways. BMMCs were stimulated with insulin (100 nM), DNP–HSA (1 ng/ml) or simultaneously with insulin and DNP–HSA (100 nM/1 ng/ml) and postnuclear lysates were subjected to immunoprecipitation with anti-Lyn (A), anti-Syk (B), anti-Fyn (C) and anti-Gab2 (D) antibodies. Western blots were probed with an anti-phospho-tyrosine specific antibody to determine activation induced phosphorylation of tyrosine residues. Loading was controlled by incubation of the blots with antibodies specific for Lyn, Syk, Fyn and Gab2. Blots are indicative of three independent experiments with similar results. Fold induction (mean $\pm$ SD) normalized to signal for loading was determined by densitometry. *p* values for DNP vs. DNP/insulin were for Lyn: 0.4, Syk: 0.11, Fyn: 0.14 and Gab2: 0.01.

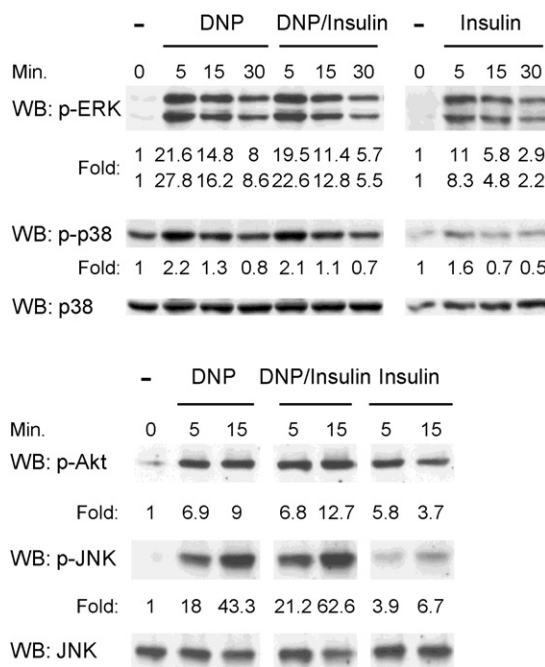


Fig. 3. Activation of Akt and MAPKs in response to insulin and FcεRI ligation in BMMCs. BMMCs were stimulated for the indicated times with insulin (100 nM), DNP-HSA (1 ng/ml) or simultaneously with insulin and DNP-HSA (100 nM/1 ng/ml) and postnuclear supernatants were analyzed in parallel for phosphorylation of ERK1/2 and p38 (upper panel) and Akt and JNK (lower panel) by Western blotting with the corresponding phosphospecific antibodies. The two sets of lanes in the lower panel represent the same experiment with cells stimulated simultaneously and then processed in parallel. Membranes were re-probed with antibodies specific for JNK and p38 respectively to control for loading. Fold induction normalized to signal for loading was determined by densitometry. Results are indicative of three independent experiments with similar results.

family, may be the main targets within the upstream signaling pathways activated through simultaneous stimulation of BMMCs with insulin and antigen.

3.3. MAP kinase activation in response to FcεRI ligation in the presence of insulin

FcεRI crosslinking induces phosphorylation and activation of the MAP kinases ERK, p38 and JNK in mast cells (Hirasawa et al., 1995; Kinet et al., 1996). In BMMCs, insulin treatment induced the phosphorylation of p38 in the absence of ERK phosphorylation, whereas ERK phosphorylation has been detected in other cells (Lessmann et al., 2006; Ray and Sturgill, 1987). There are no data available about insulin-mediated JNK activation in mast cells. The phosphorylation status of ERK, p38 and JNK in BMMCs after FcεRI crosslinking was examined by Western blotting with antibodies that recognize selectively these kinases in their phosphorylated state (Fig. 3). ERK phosphorylation after incubation of BMMCs with insulin was readily detectable in contrast to published results (Lessmann et al., 2006), and was weaker when compared to the signal after FcεRI crosslinking but followed the same time course with a peak after 5 min of stimulation and a decline thereafter over time. Combined stimulation did not augment ERK phosphorylation (Fig. 3, upper panel). Similar results were found for p38 phosphorylation (Fig. 3, upper panel). Insulin treatment resulted in the phosphorylation of JNK (Fig. 3, lower panel), which was considerably weaker than after incubation with antigen alone. When BMMCs were stimulated with both, insulin and antigen, JNK phosphorylation was further increased over the time course tested as compared to cells incubated with antigen alone. These results suggest a synergistic activity of insulin not only in immediate effector

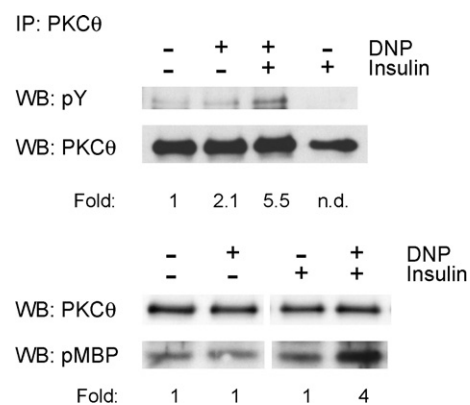


Fig. 4. Enhanced PKCθ kinase activity in BMMCs after FcεRI ligation in the presence of insulin. BMMCs were stimulated for the indicated times with insulin (100 nM), DNP-HSA (1 ng/ml) or simultaneously with insulin and DNP-HSA (100 nM/1 ng/ml) and PKCθ was immunoprecipitated from postnuclear supernatants. Tyrosine phosphorylation of PKCθ was determined with an anti-phospho-tyrosine specific antibody (upper panel). For kinase assay, immunoprecipitates were incubated in kinase buffer containing MBP as substrate. Kinase activity was determined by immunoblotting of MBP with a phospho-MBP specific antibody (lower panel). Western blotting using anti-PKCθ antibody was used for loading control. Fold induction normalized to signal for loading was determined by densitometry. Results are indicative of three independent experiments with similar results. n.d. not detectable.

functions in mast cells after antigen stimulation but also in JNK activation further downstream that may lead to synthesis and release of cytokines and lipid mediators.

3.4. Activation of Akt in BMMCs stimulated with insulin and antigen

Gab2 has been reported to have a role in the regulation of PI3K and activation of Fyn is required for the phosphorylation of the downstream serine/threonine kinase, Akt in mast cells (Gu et al., 2001; Parravicini et al., 2002). Furthermore, the activation of PI3K and Akt by insulin is critical for increased glucose transport (Shepherd et al., 1995). We therefore tested the phosphorylation of Akt in BMMCs stimulated with antigen in the presence of insulin. We found that indeed co-stimulation of BMMCs with antigen and insulin resulted in an increased and more sustained activation of Akt compared to BMMCs stimulated with antigen or insulin alone (Fig. 3 lower panel). These results suggest that insulin enhances antigen-mediated activation of the PI3K pathway.

3.5. Increased activation of PKCθ in response to co-stimulation with insulin and antigen

PKCθ, a calcium independent PKC isoform, was proposed to have a role in signaling events downstream of FcεRI ligation. PKCθ was shown to directly associate with Lyn upon antigen stimulation and enhanced degranulation was detected when PKCθ was over expressed in RBL-2H3 cells (Liu et al., 2001). Furthermore, in skeletal muscle, a major target for insulin, PKCθ has been proposed to be involved in defective receptor mediated activation of the signaling cascade leading to insulin resistance (Serra et al., 2003). To determine whether PKCθ was activated both downstream of FcεRI ligation and insulin stimulation a PKCθ protein kinase assay was performed with lysates obtained from BMMCs treated with either insulin or antigen alone or in combination (Fig. 4). Whereas insulin alone did not result in PKCθ activation, the combined treatment resulted in a strong activation of PKCθ demonstrated both by enhanced tyrosine phosphorylation (Fig. 4, upper panel) and increased kinase activity (Fig. 4, lower panel). Crosslinking of FcεRI in the absence of insulin resulted in increased phosphorylation of

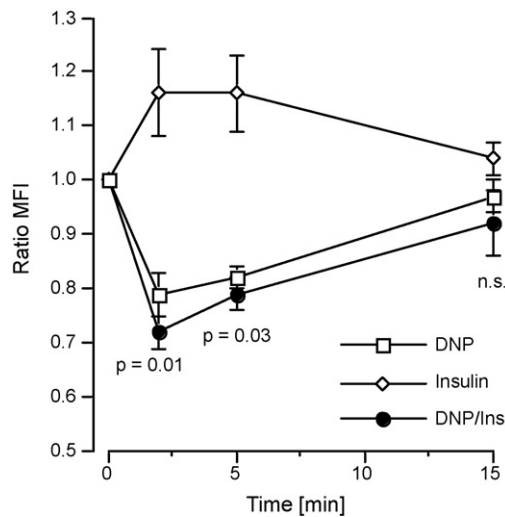


Fig. 5. Determination of cellular F-actin in BMMCs. IgE-sensitized BMMCs were stimulated with either insulin, DNP-HSA or simultaneously with insulin and DNP-HSA for the indicated time. Cells were then fixed, permeabilized, stained with phalloidin-TRITC, and analyzed by FACS. Results represent mean $\pm$ SD of three experiments. For indicated *p* values results from DNP vs. DNP/Ins were compared. Statistical analysis was performed using the Student's *t* test.

PKC θ but not enhanced kinase activity when compared to unstimulated BMMCs indicating that phosphorylation on tyrosine residues alone was not sufficient to detect increased kinase activity above background at the concentration of antigen used. Augmented PKC θ kinase activity was observed (9-fold over background) when BMMCs were stimulated with 100 ng/ml DNP-HSA but was not further increased when stimulated simultaneously with insulin and antigen (not depicted).

3.6. F-actin dynamics after BMMC treatment with insulin

PKC θ has been shown to be involved in the rearrangement of F-actin in T cells (Sasahara et al., 2002). Fc ϵ RI signaling induces changes in cellular F-actin content in mast cell lines (Kettner et al., 2004; Pivniouk et al., 2003). Furthermore, it has been shown that insulin induces F-actin rearrangement in adipocytes (Wang et al., 1998). We therefore measured F-actin content in BMMCs stimulated with either insulin and antigen alone or in combination. Insulin stimulation induced actin polymerization as after two and 5 min of stimulation an increase in F-actin content was detected (Fig. 5). Fc ϵ RI ligation resulted in an initial decrease in F-actin content, with the maximum decrease being observed after two min of stimulation. Subsequently, F-actin content slowly increased but was still lower than baseline 15 min after Fc ϵ RI ligation. When BMMCs were incubated with both, insulin and antigen, cellular F-actin content was further reduced after two min of incubation compared to treatment with DNP-HSA alone and remained so at all time points tested. These results suggest that disassembly or severing of F-actin is one of the mechanisms involved in enhanced mast cell degranulation induced after antigen-mediated stimulation in the presence of insulin.

4. Discussion

Reports coming forth with new functions for mast cells to regulate and influence both innate and adaptive immunity trigger the interest to study receptors for ligands that stimulate mast cells by their own specific signaling cascades or may modulate Fc ϵ RI-mediated mast cell activation (Gomi et al., 2000; Jawdat et al., 2006; Kuehn et al., 2008; Maurer et al., 2004; Metz et al., 2006). Here we

present results that provide insight to the molecular mechanisms downstream of such a receptor, the insulin receptor. Recently it has been proposed that mast cells might have a role in the development of type I diabetes, either directly by nourishing the inflammation of the tissue or in an indirect role by modulating T regulatory cell responses (Geoffrey et al., 2006; Lu et al., 2006). Reports of direct impact of insulin on mast cell responses merit the study of the molecular mechanisms underlying insulin induced signaling alone or in the context of an IgE-mediated response (Cavalher-Machado et al., 2004; Diaz et al., 1996; Vianna and Garcia-Leme, 1995).

We studied the signaling pathways involved in insulin-mediated augmentation of mast cell mediator release after Fc ϵ RI ligation. Our results suggest that insulin may modulate IgE-mediated degranulation and subsequent mediator release by enhanced activation of both the primary and complementary pathways downstream of Fc ϵ RI. An indication for insulin to fulfill such a role came from the experiments where we sought to determine the phosphorylation state of the Src family kinases Lyn and Fyn which together with Syk are transmitting the very early signaling events evoked by crosslinking of Fc ϵ RI. Although insulin alone stimulated tyrosine phosphorylation of all three kinases, no degranulation was observed, suggesting that either a signaling event necessary to trigger granule exocytosis was absent or the required signaling threshold was not reached. Indeed, in BMMCs, insulin treatment did not provoke any measurable increase in intracellular calcium concentration, an indication that the threshold for sufficient calcium mobilization required for degranulation was not reached (Lessmann et al., 2006). Enhanced phosphorylation of Lyn and Fyn upon simultaneous stimulation with insulin and antigen suggests that insulin-mediated stimulation of Syk and especially of Lyn and Fyn may feed directly into the two signaling pathways that cooperate to promote degranulation upon Fc ϵ RI engagement (Parravicini et al., 2002).

The pathway mediated by Lyn via downstream activation of Syk/LAT/SLP-76 leading to calcium mobilization (Pivniouk et al., 1999; Saitoh et al., 2000; Silverman et al., 2006) seems not to be the major pathway through which insulin stimulation augments IgE-dependent mast cell activation. Our results that Syk phosphorylation was not increased upon co-stimulation with insulin and antigen, together with the finding that insulin stimulation alone does not lead to calcium mobilization (Lessmann et al., 2006) support such a conclusion. Nevertheless, an increase of intracellular calcium after co-stimulation is suggested by our observation of a greater decrease of F-actin in the initial, calcium influx dependent, phase of stimulation (Kettner et al., 2004). A possible explanation could be the activation of Sphingosine kinases 1 and 2 (SphK1 and SphK2) leading to S1P generation and a possible amplification of the calcium response (Choi et al., 1996). Optimal SphK1 and SphK2 activation requires both the activation of Lyn as well as the Fyn/Gab2/PI3K pathway (Olivera et al., 2006). Our observation of modestly enhanced Lyn phosphorylation together with increased phosphorylation of Fyn and Gab2 may support such a hypothesis. In BMMCs, Gab2 is essential for PI3K recruitment and activation after Fc ϵ RI ligation (Gu et al., 2001) and our observation that Akt phosphorylation is increased after combined stimulation with insulin and antigen is consistent with enhanced PI3K activation. Similar results have recently been reported where a role for insulin in promoting mast cell survival via PI3K activation was proposed (Lessmann et al., 2006).

PI3K activation leads to the recruitment of pleckstrin homology domain-containing phosphatidylinositol-dependent protein kinase 1 (PDK1) (Currie et al., 1999). The later requires both Fyn and Gab2 for activation and has been shown to phosphorylate Akt and PKC θ (Alessi et al., 1997; Anderson et al., 1998; Gu et al., 2001; Lee et al., 2005; Parravicini et al., 2002). PKC θ has been reported to have a role in insulin signaling (Donnelly et al., 1994;

Yamada et al., 1995) as well as in Fc ϵ RI-mediated signal transduction through its interaction with Lyn (Liu et al., 2001). PKC θ is a member of the calcium independent novel PKC subfamily (Baier et al., 1993). These characteristics let us hypothesize that PKC θ might be involved in insulin enhanced Fc ϵ RI signaling. Our results suggest that indeed PKC θ is strongly activated when BMMCs were stimulated with antigen in combination with insulin. This activation was evident both through enhanced tyrosine phosphorylation as well as strongly increased kinase activity. It is tempting to speculate that increased Lyn activity might explain augmented tyrosine phosphorylation which together with strong PI3K/PDK1 activation would lead to a much greater kinase activity as when cells were stimulated with antigen or insulin alone. Enhanced PKC θ activation may furthermore, through activation of the Carma1/Malt1/Bcl10 complex (CARD, membrane-associated guanylate kinase, MAGUK, protein 1/mucosa-associated lymphoid tissue lymphoma translocation protein 1/B cell lymphoma 10), translate to increased NF- κ B activation and subsequent cytokine production (Klemm et al., 2006).

PKC θ may link Fc ϵ RI signaling to the cytoskeleton given its role in actin reorganization in T cells where it has been shown to phosphorylate Wiskott–Aldrich syndrome protein-interacting protein (WIP) leading to subsequent Wiskott–Aldrich syndrome protein (WASP) mediated increase of actin polymerization (Sasahara et al., 2002). We only observed a modestly accelerated increase of F-actin in the polymerization phase in BMMCs treated with antigen in the presence of insulin. These results suggest that enhanced PKC θ activity above normal did not measurably translate to accelerated actin polymerization unlike what we observed in BMMCs lacking the PKC θ substrate WIP (Kettner et al., 2004). Nevertheless, the increase of F-actin upon insulin stimulation indicates that insulin-mediated signaling leads to actin cytoskeletal rearrangements in BMMCs. Similar results have been reported for adipocytes (Jiang et al., 2002). Enhanced PKC θ activation may not only regulate cytoskeletal rearrangement but also lead to increased JNK activation, a concept that is supported by studies in T cells where PKC θ selectively stimulates JNK activity (Avraham et al., 1998). Furthermore, JNK phosphorylation is reduced in WIP and WASP deficient mast cells indicating a role of actin cytoskeletal regulation of JNK (Kettner et al., 2004; Pivniouk et al., 2003). Together these findings strengthen a possible role for PKC θ in the signaling pathway to both actin dynamics and JNK activation in BMMCs. Alternatively, it is also possible that signaling may bifurcate earlier as JNK activation is severely diminished in BMMCs lacking either SLP-76, Fyn or Gab2 (Gomez et al., 2005; Gu et al., 2001; Pivniouk et al., 1999).

Insulin dependent signals play important roles for the regulation of proliferation, differentiation and metabolism in various cells, including hematopoietic cells (Farrar et al., 1990; Saltiel and Kahn, 2001). Our findings that insulin activates the MAP kinases JNK, ERK and p38 agree with such a role for insulin in BMMCs. Whereas JNK phosphorylation was evidently increased after Fc ϵ RI ligation together with insulin stimulation, ERK and p38 phosphorylation were not augmented. This suggests, that insulin selectively enhances the signaling pathway leading to JNK activation but has no effect on those of the other MAP kinases. In contrast to the study of Lessmann et al. (2006) we readily detected ERK phosphorylation after stimulation with insulin. Our results are in agreement with studies in other cell types where ERK activation has been described after insulin treatment (Ray and Sturgill, 1987). Differences in mouse strains from which BMMCs were generated with dissimilar insulin sensitivity may explain the contrasting findings (Goren et al., 2004; Yamashita et al., 2007). Still, enhanced degranulation upon combined stimulation with antigen and insulin was observed in BMMCs derived from either mouse strain. From these results a major contribution of ERK to the enhanced degranulation response might be excluded.

There is very little literature about insulin receptor (IR) expression on mast cells in general and mouse mast cells in particular with one report about expression on rat peritoneal mast cells (Pallinger and Csaba, 2005). Evidence for the presence of IRs on mouse BMMCs stems from the study were insulin and insulin growth factor-1 (IGF-1) promote BMMCs survival (Lessmann et al., 2006). Insulin binds to two IR isoforms (IRa and IRb), which are a result from tissue-specific alternative splicing. Furthermore, insulin can also bind to IGF-1 receptor (IGF1R) as well to heterodimers composed of two different receptor monomers from IGF1R and IRs (Nakae et al., 2001; White, 2003). It is not clear yet whether downstream signaling after insulin ligation of different receptors varies in quality or quantity and it remains to be established through which receptor insulin elicits the here reported responses in mast cells. Our finding that co-stimulation of insulin was strongest at higher insulin concentrations may indicate that insulin not only signals through IRs but also through IGF1R and possibly heterodimeric receptors, which have a lower binding affinity for insulin (Soos et al., 1993), contributed to the signaling events presented here. Therefore data presented here might result from the sum of signals mediated by all receptors that bind insulin. It is noteworthy, that BMMCs do not express the major IR signal transducing proteins, insulin receptor substrate-1 and -2 leaving the immediate signal transmitting substrate of the IR elusive (Welham et al., 1997).

The here presented molecular evidence of the impact of insulin on mast cell function adds its receptor to the list of mast cell specific receptors that are capable of enhancing Fc ϵ RI-mediated mast cell activation utilizing “alternative” mechanisms to integrate the required signaling cascades. We can assume that mast cells in the presence of insulin might produce a more robust inflammatory response in conjunction with antigen. Our findings may contribute to the understanding of how the complex signaling cascades in mast cells might be tuned in order to develop new strategies for the treatment of disorders with mast cell involvement.

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