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Selective Activation of Fyn/PI3K and p38 MAPK Regulates IL-4 Production in BMMC under Nontoxic Stress Condition

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Mast cells have the ability to react to multiple stimuli, implicating these cells in many immune responses. Specific signals from the microenvironment in which mast cells reside can activate different molecular events that govern distinct mast cells responses. We previously demonstrated that hydrogen peroxide (H₂O₂) promotes IL-4 and IL-6 mRNA production and potentiates FcεRI-induced cytokine release in rat basophilic leukemia RBL-2H3 cells. To further evaluate the effect of an oxidative microenvironment (which is physiologically present in an inflammatory site) on mast cell function and the molecular events responsible for mast cell cytokine production in this environment, we analyzed the effect of H₂O₂ treatment on IL-4 production in bone marrow-derived, cultured mast cells. Our findings show that nanomolar concentrations of H₂O₂ induce cytokine secretion and enhance IL-4 production upon FcεRI triggering. Oxidative stimulation activates a distinct signal transduction pathway that induces Fyn/PI3K/Akt activation and the selective phosphorylation of p38 MAP kinase. Moreover, H₂O₂ induces AP-1 and NFAT complexes that recognize the IL-4 promoter. The absence of Fyn and PI3K or the inhibition of p38 MAPK activity demonstrated that they are essential for H₂O₂-driven IL-4 production. These findings show that mast cells can respond to an oxidative microenvironment by initiating specific signals capable of eliciting a selective response. The findings also demonstrate the dominance of the Fyn/p38 MAPK pathway in driving IL-4 production. The Journal of Immunology, 2007, 178: 2549–2555.
as a stimulatory and regulatory factor in B cell growth, CD4+ Th2 polarization, isotype switching, and IgE production (15, 16). IL-4 also modulates inflammatory reactions and could be produced by stimulation of BMMC with low doses of Ag (7). In this study we find that H2O2 induces IL-4 production in BMMC through preferential activation of a distinct signal transduction pathway that includes Fyn/P13K/Akt activation, the selective phosphorylation of p38 MAPK, and the DNA binding of NFAT and AP-1, which are both known to modulate IL-4 gene transcription (17). Moreover, the results show that both Fyn and p38 are critical for H2O2-driven IL-4 production. Thus, as seen with a weak stimulus (7), oxidative treatment evokes specific mast cell responses that cause selective gene expression.

Materials and Methods

Antibodies and reagents

Murine DNP-specific IgE was produced as described (18). DNP-human serum albumin (HSA) (DNP₃₆-HSA; Ag) was from Sigma-Aldrich. Abs used for immunoprecipitation were anti-Lyn, anti-Fyn, anti-P13K, and anti-Akt, all from BD Biosciences. A peroxidase-conjugated anti-phosphotyrosine Ab (Cell Signaling) was used to detect phosphorylated proteins in the immunoblotting experiments. Rabbit Abs to ERK2, JNK1, and p38 MAPK were from Santa Cruz Biotechnology, and mouse anti-phospho-p38 MAPK were from Cell Signaling. The secondary Abs used for immunoblotting were goat anti-mouse IgG-HRP and goat anti-rabbit IgG-HRP from Sigma-Aldrich. PD98059, a specific inhibitor of ERK kinase, and SB203580, a specific inhibitor of p38 kinase, were purchased from Calbiochem. All other reagents were purchased from Sigma-Aldrich.

Mice and cell culture

Fyn⁻/⁻ mice (strain B6.129S-Fyn) and Lyn⁻/⁻ mice (strain c57BL/6J-Lyn(N6)) were from The Jackson Laboratory. P13K-deficient mice, which are on a 129p inbred genetic background, were generated as described previously (19). Wild-type age- and sex-matched litters were used as controls. All mice were used in accordance with National Institutes of Health guidelines and National Institute of Arthritis and Musculoskeletal and Skin Diseases-approved Animal Study Proposal A001-04-03. BMMC were obtained by spleen cell dissociation, incubation with FcγR blocking Abs, and cultured in RPMI 1640 supplemented with 10% FCS, 1% L-glutamine, 100U/ml penicillin, 100 μg/ml streptomycin, nonessential amino acids, sodium pyruvate, and HEPES buffer containing 20 ng/ml stem cell factor (SCF) and 4 ng/ml IL-3 for 4 to 8 wk. All cell cultures were grown at 37°C in a humidified atmosphere with 5% CO₂.

Cell culture reagents were obtained from BioWhittaker. After 4 wk, 1 × 10⁶/ml BMMC were stained for FcγR using 0.01% Nonidet P-40 lysis buffer. Lysates were then centrifuged for 5 min at 1200 rpm and the supernatant was used. Total RNA was isolated from 5 × 10⁶ cells using TRIzol Reagent (Invitrogen Life Technologies) according to manufacturer’s instructions.

Preparation of protein extracts

Cell stimulation

Because SCF and IL-3 induce some cell responses, BMMC were rested before stimulation by the removal of SCF overnight (for 16 h) followed by the removal of IL-3 for 3 h. Then cells were incubated with the appropriate H₂O₂ concentration in SCF and IL-3-free media. To stimulate cells via FceRI, cells were sensitized with 1 μg/ml anti-DNP mouse IgE in SCF and IL-3-free medium for 4 h and then washed twice with PBS. Culture medium containing the 100 ng/ml DNP₁₀-HSA (Ag) was then added. At the indicated times, samples were collected by centrifugation for 5 min at 1200 rpm and RNA or protein was extracted.

RT-PCR

Total RNA was isolated from 5 × 10⁶ cells using TRIzol Reagent (Invitrogen Life Technologies) according to manufacturer’s instructions. cDNA was synthesized from total RNA by reverse transcription using Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies) and oligo(dT)ₙ priming. cDNA was amplified by PCR using Red Taq polymerase (Sigma-Aldrich) in a GeneAmp PCR system 2400 thermal cycler (PerkinElmer). Amplification conditions were as follows: hot start at 94°C for 5 min, denaturation at 94°C (20 s), annealing at 55°C (15 s) for primers IL-4, IL-5, and IL-10 or 60°C (15 s) for GAPDH and IL-6, extension at 72°C (45 s) for 35 cycles, and a final extension step at 72°C for 10 min. All primers for mouse cytokines were purchased from Clontech. The PCR products were separated by agarose gel electrophoresis and were visualized by ethidium bromide staining. A 100-bp DNA ladder (MBI Fermentas) was run as a reference marker. Gel documentation and subsequent densitometric analysis of band intensity was conducted using the Gel Doc 2000 system (Bio-Rad).

Cytokine release detection

Quantitative measurement of IL-4 in cell supernatants was performed using the BioTrak mouse IL-4 ELISA from Amersham Biosciences. Fifty microliter aliquots of cell supernatants were used for the assay according to the manufacturer’s instructions.

Electromobility shift assay

The sequence of the top strand of double-stranded oligonucleotide probes used in EMASs were 5′-CTTTTGCCTATCAAAATCTTCCACACTTAC-3′ for NFAT and 5′-CGCTTGATGAGTCAGCCGGAA-3′ for AP-1. Oligonucleotides were end labeled with [γ-³²P]dATP by incubation with T4 polynucleotide kinase (MBI Fermentas), annealed, and purified on column SpinX Costar #160 (Corning) filled with Sephadex G-50 fine (Amersham Biosciences). Nuclear extracts (10 μg) were incubated with 0.5–1 ng of labeled probe (10,000–20,000 cpm) for 30 min at room temperature in binding buffer (200 mM HEPES (pH 7.9), 500 mM KCl, 10 mM MgCl₂, 1 mM EDTA, 50 mM PMSF, and 0.5 mM DTT). The probes were resolved by SDS-PAGE and Western blot analysis.
transcription factor CREB, which is not modified in its binding activity following the cell treatment used in this study (data not shown).

Data analysis

The data shown is a summary from at least three experiments. Data are shown as the mean ± SD. Statistical significance of the data was calculated using a Student t test.

Results

H$_2$O$_2$ treatment induces IL-4 up-regulation

Mast cells are known to produce a variety of cytokines in response to FcεRI stimulation. We previously demonstrated that H$_2$O$_2$ treatment can also cause cytokine gene expression in the rat basophilic leukemia RBL-2H3 cell line (14), but this had not been tested in BMMC.

We first compared the profile of cytokine mRNA from unstimulated and cells stimulated via FcεRI or with 10 nM H$_2$O$_2$. As shown in Fig. 1A, a nanomolar concentration of H$_2$O$_2$ clearly increased IL-4 mRNA while weakly affecting IL-5 and IL-6 mRNA levels and had no effect on IL-10 mRNA levels. The IL-4 response to H$_2$O$_2$ was similar to that of FcεRI stimulation.

Recently, it was proposed that at inflammatory sites oxidative stress might induce IL-4 production by mast cells and basophils and direct Th2 cell differentiation (21). Importantly, non-IgE-dependent modes of maintaining Th2 responses have been demonstrated, as FcεRI-null mice develop a normal Th2 response when infected with Schistosoma mansoni despite lacking FcεRI dependent IL-4 production (22). Thus, the oxidative microenvironment might provide such an alternate mode by promoting IL-4-production by mast cells in an IgE-independent manner. So, BMMC were stimulated with varying concentrations of H$_2$O$_2$, and IL-4 mRNA expression was evaluated by RT-PCR. To also determine whether H$_2$O$_2$ stimulates IL-4 secretion, we measured the IL-4 concentrations in the culture supernatants of BMMC incubated with varying doses of H$_2$O$_2$ for 24 h. The results shown in Fig. 1, B and C, demonstrate that H$_2$O$_2$ treatment (at 10 nM) increased IL-4 mRNA by >3-fold relative to the untreated sample (Fig. 1B). Consistent with this observation, the stimulation of BMMC with varying doses of H$_2$O$_2$ induced the secretion of IL-4 reaching a maximum at 10 nM of 19 ± 2.3 pg/10$^6$ cells, an increase approximating 3-fold more than that observed in the medium of unstimulated cells (Fig. 1C). As a positive control, we stimulated FcεRI-dependent IL-4 secretion and found it to be ~2-fold greater than H$_2$O$_2$-induced secretion (Fig. 1D). Concomitant stimulation via FcεRI and H$_2$O$_2$ treatment demonstrated an additive effect on IL-4 secretion (Fig. 1D), consistent with our previous results (14) and suggesting that the signals leading to IL-4 synthesis and secretion were not saturated by either individual stimulus.

H$_2$O$_2$ treatment causes phosphorylation of multiple signaling proteins used by FcεRI but does not stimulate LAT phosphorylation

To assess which signals are required for H$_2$O$_2$-mediated cytokine production, we studied the tyrosine phosphorylation of various signaling proteins known to function downstream of FcεRI. Lyn, Fyn, LAT, PI3K, and Akt were immunoprecipitated and their phosphorylation states were determined by immuno blotting with a mAb to phosphotyrosine. Equal protein loading was verified by immuno blotting with an Ab to the specific protein and all results were normalized to the total protein following densitometric analysis. As shown in Fig. 2A, FcεRI stimulation of BMMC caused increased tyrosine phosphorylation of Lyn, LAT, Fyn, PI3K, and Akt. The results following H$_2$O$_2$ treatment of BMMC were quite similar with one significant exception, namely the failure of LAT to become phosphotyrosinated (Fig. 2A). The kinetic experiments on H$_2$O$_2$-treated cells shown in Fig. 2B demonstrate that Fyn and Lyn tyrosine phosphorylation are rapid (within 5 min of stimulation). Maximal phosphorylation of PI3K and Akt lags behind Lyn and Fyn phosphorylation but appears to still be augmented after 15 min of treatment. H$_2$O$_2$ failed to induce LAT phosphorylation at any time point studied (Fig. 2B). Thus, these results show that unlike FcεRI stimulation, which results in robust LAT phosphorylation and generates downstream signals (see Fig. 4 below), an oxidative stimulus fails to activate LAT. In contrast, Fyn activation and the phosphorylation of PI3K and Akt were robust (Fig. 2B). Interestingly, we previously observed similar results by weak stimulation of BMMC (7).

H$_2$O$_2$-induced IL-4 up-regulation requires Fyn

Cytokine production in mast cells as a consequence of FcεRI stimulation seems to be primarily driven by Fyn and not Lyn kinase (23, 24). In fact, FcεRI-dependent stimulation of IL-4 production was demonstrated to be defective in Fyn-deficient BMMC but not in Lyn deficiency (23–25). To determine whether Lyn and/or Fyn might be required for H$_2$O$_2$-induced IL-4 production, we analyzed IL-4 mRNA up-regulation using BMMC from Lyn$^{-/-}$, Fyn$^{-/-}$, and PI3K$^{-/-}$ mice. BMMC from wild type, Lyn-, Fyn-, and PI3K-null mice were treated with 10 nM H$_2$O$_2$ for 3 h and then analyzed...
together with untreated samples for IL-4 mRNA production. Fig. 3 shows that H$_2$O$_2$ induced up-regulation of IL-4 mRNA only in wild-type and Lyn-null BMMC. In contrast, H$_2$O$_2$ failed to induce a significant increase in IL-4 mRNA in both Fyn- and PI3K-null BMMC. The results were also confirmed by ELISA (data not shown). These findings are consistent with the requirement for Fyn in Fc$\gamma$RI-induced IL-4 production (23) and demonstrate that H$_2$O$_2$-driven IL-4 production preferentially uses Fyn-mediated signals, whereas Lyn-mediated signals are not crucial. Moreover, these results indicate that PI3K is a key regulatory factor for IL-4 expression.

**H$_2$O$_2$ induce preferentially p38 MAPK phosphorylation**

H$_2$O$_2$ stimulation is known to promote the activation of MAPK pathways, including ERK, JNK, and p38 kinase in various cell types (26). Because MAPKs play an important role in the regulation of cytokine production in mast cells (27), we sought to determine whether H$_2$O$_2$ treatment of BMMC led to the activation of MAPKs. Cells were stimulated with varying concentrations of H$_2$O$_2$ and the degree of MAPKs phosphorylation was compared with cells stimulated via Fc$\gamma$RI. As shown in Fig. 4A, H$_2$O$_2$ treatment did not induce the phosphorylation of ERK and JNK; however, as expected ERK and JNK were potently activated after Fc$\gamma$RI engagement. In contrast, p38 MAPK phosphorylation was significantly induced in a dose-dependent manner by H$_2$O$_2$ treatment. At 10 nM, H$_2$O$_2$ phosphorylation of p38 MAPK reached levels of ~60% of that seen with Fc$\gamma$RI stimulation. Because Fyn kinase was reported to stimulate p38 MAPK phosphorylation (7, 23), we investigated whether Lyn or Fyn might be required for p38 MAPK phosphorylation following H$_2$O$_2$ treatment of BMMC deficient in these Src kinases. Fig. 4B demonstrates that both Fc$\gamma$RI stimulation and H$_2$O$_2$ treatment induce p38 MAPK activation in wild-type and Lyn-null BMMC. In contrast, p38 MAPK activation was dramatically inhibited in Fyn-null BMMC because both Fc$\gamma$RI stimulation or oxidative treatment failed to induce its phosphorylation. These results establish that H$_2$O$_2$ treatment of BMMC activates Fyn-dependent phosphorylation (activation) of p38 MAPK.

It is well understood that multiple pathways cooperate with the activity of MAPKs in gene expression (28). Thus, to determine the importance of p38 MAPK activity in the mRNA accumulation of
IL-4 induced by H₂O₂ stimulation, we used pharmacological inhibitors of ERK (PD98059) and p38 MAPK (SB203580) activity. Cells were briefly pretreated with these inhibitors as previously described (7) and were then stimulated via FcɛRI or by H₂O₂ treatment. Fig. 5 shows that the treatment of BMMC with the two MAPK inhibitors caused a marked decrease in both FcɛRI- and H₂O₂-induced IL-4 mRNA accumulation. In fact, after treatment with the p38 MAPK inhibitor SB203580 the level of IL-4 mRNA in all samples appeared to be reduced below those of nontreated cells, while the ERK inhibitor PD98059 showed only weak effect on both FcɛRI- and H₂O₂-stimulated samples, but some IL-4 mRNA was still detected (Fig. 5). Results were also confirmed by ELISA (data not shown). These findings indicate that p38 MAPK activation is essential for IL-4 production whereas ERK has a minimal role. The findings are consistent with the preferential signaling of p38 MAPK in the induction of an IL-4 gene in cells exposed to oxidative stimulation.

**H₂O₂ treatment induces DNA binding of NFAT and AP-1 on the IL-4 promoter**

IL-4 gene expression in mast cells is selectively regulated by specific transcription factors (16). In T cells as well as in mast cells the NFAT and AP-1 transcriptional factors bind cooperatively to their cognate binding sites in the IL-4 promoter, and the binding of both is necessary for maximal transcription in T cells (29). Because NFAT and AP-1 are known to be redox-sensitive transcription factors, we assessed their DNA binding activity in unstimulated and H₂O₂-treated BMMC using EMSAs. As an NFAT probe, the sequence between −88 and −60 in the 5’ region of the IL-4 gene was used (the activation-responsive element), which was demonstrated to be a binding site for NFAT but not for AP-1 in mast cells (17). To determine AP-1 DNA binding activity, a commercial probe containing the consensus binding site for AP-1, present in the IL-4 promoter (29, 30), was used. Fig. 6A shows that H₂O₂ treatment caused an increase in both AP-1 and NFAT DNA binding activity, evidenced by a shift of the NFAT band and the

**FIGURE 4.** H₂O₂ treatment of BMMC induces preferential phosphorylation of p38 MAPK. A. BMMC were stimulated with the indicated concentrations of H₂O₂ for 30 min. As a positive control, DNP-specific IgE-sensitized BMMC were stimulated with 100 ng/ml DNP-HSA. p38 MAPK, ERK, and JNK phosphorylation (p-) was measured by Western blot using specific anti-phospho-p38, anti-phospho-ERK, and anti-phospho-JNK. B. BMMC from wild-type, Lyn⁻/⁻, and Fyn⁻/⁻ mice were stimulated via FcɛRI or with 10 nM H₂O₂. p38 MAPK phosphorylation was evaluated as in A. Asterisks indicate statistical difference (*, p < 0.006; **, p < 0.002) vs untreated cells.

**FIGURE 5.** The selective inhibition of p38 MAPK prevents IL-4 mRNA up-regulation following H₂O₂ treatment of BMMC. BMMC were preincubated with 10 μM PD98059 or 10 μM SB203580 for 30 min followed by IgE/DNP or H₂O₂ stimulation for 3 h or left untreated. IL-4 mRNA expression was evaluated by RT-PCR after isolation of total RNA. GAPDH was used to normalize the data. The asterisk indicates statistical difference (*, p < 0.001) vs unstimulated cells.
increased intensity of the AP-1 band. The increase in binding activity was similar to that induced by FcεRI stimulation. The specificity of binding was also confirmed by incubation with a 100-fold excess of unlabeled oligonucleotide.

To further explore whether the H₂O₂-mediated activation of p38 MAPK plays a role in the DNA binding activity of NFAT and AP-1, BMMC were preincubated with the p38 inhibitor SB203580 and were subsequently stimulated for 1 h in the presence or absence of 10 nM H₂O₂. The nuclear extracts were then assayed to determine the DNA binding activity of AP-1 and NFAT. Fig. 6B shows that inhibition of p38 MAPK activity prevents the binding of AP-1 and NFAT to their consensus binding domains in the IL-4 promoter. These findings link the H₂O₂-induced activation of p38 MAPK as essential for IL-4 gene expression.

**Discussion**

ROS are important determinants in the regulation of cell function. This includes proliferation, apoptosis, transformation, and the mediation of cellular responses by various extracellular stimuli (31). Several lines of evidence suggest a role for ROS in the production of cytokines, growth factors, and hormones and the activation of nuclear transcription, although the mechanisms are still unclear (31). Probably, ROS exerts a direct effect on kinases or redox-sensitive proteins by inducing conformational changes that are required for protein activation and these early signals can then elicit transcriptional activity (32). In all biological systems H₂O₂ is a physiological product and is well documented as influencing mast cell behavior, although the findings are often contradictory about the beneficial or detrimental effects of H₂O₂ on mast cell effector responses (33, 34).

In the present study we find that low concentrations of H₂O₂, known not to alter cell growth or apoptosis (14) or to significantly promote cell degranulation (our unpublished data) can induce IL-4 production in mast cells independently of other stimuli and, together with FcεRI stimulation, further enhance IL-4 production. We demonstrate that H₂O₂-mediated induction of IL-4 is dependent on Fyn kinase and p38 MAPK activity, which drives both AP-1 and NFAT binding to their respective consensus regions on the IL-4 promoter. This appears to be independent of a requirement for the Lyn kinase activity, LAT phosphorylation, and is primarily independent of ERK and JNK because these MAP kinases were not potently stimulated by H₂O₂ treatment.

Our findings are in agreement with several previous studies that demonstrated the involvement of PI3K/p38 activated pathway, FcεRI-induced IL-4 production in the mast cell (23, 35). It is known that PI3K regulates the activation of Rac, which, in turn, activates p21-activated kinase resulting in the induction of p38 MAPK and JNK (36). Moreover, the Fyn kinase is known to be required for PI3K activation, and Fyn deficiency results in the reduction of both p38 MAPK and JNK activation but not that of ERK (23). Additionally, the importance of MAPKs for Th2 cytokine production has been reported (37). Thus, the collective findings provide a strong argument for the role of Fyn and PI3K in regulating MAPK activity that promotes the Th2 cytokine response of mast cells.

The physiological production of IL-4 is strictly regulated and is restricted to activated T cells and mast cells/basophils as a result of the coordination and cooperation of NFAT and AP-1 on the IL-4 gene promoter (29). In both T cells and mast cells it was demonstrated that NFAT is associated with the IL-4 transcription complex and that is involved in IL-4 production (17). Although the importance of AP-1 binding is less clear, both NFAT and AP-1 are transcriptional factors known to be redox regulated. In fact, it has been previously demonstrated that AP-1 DNA binding activity was increased after 1 h of exposure to a micromolar concentration of H₂O₂, probably due to AP-1 phosphorylation by stress-inducible protein kinases like JNK or p38 MAPK (38). NFAT binding to DNA was also demonstrated to occur in an H₂O₂-dependent manner, because it was impaired by preincubation with the specific H₂O₂ scavenger catalase (39). Importantly, we now establish a connection between the H₂O₂ induction of early signaling molecules and the activation of both AP-1 and NFAT activities that promote the consequent IL-4 gene transcription and cytokine production.

In conclusion, our findings demonstrate a strong similarity between the weak stimulation of FcεRI (7) and H₂O₂ treatment. Both stimuli induced the selective activation of signal molecules, leading to a limited profile of mast cell responses (4, 7). Low doses of Ag preferentially favored the phosphorylation of Gab2, which is a consequence of Fyn activation (8), and p38 MAPK activation resulting in expression of some cytokines, among them IL-4 (7). Similarly, H₂O₂ stimulation induced the activation of Fyn with the consequent phosphorylation of PI3K and Akt and p38 MAPK.
activation, both Fyn and p38 MAPK being required for IL-4 production. It should be noted that selective responses as a consequence of weak stimulus is not a phenomenon restricted to mast cells. T cell activation through the TCR results in preferential cytokine production related to the strength of the stimulus. In fact, it was demonstrated that both weak and strong TCR stimuli induce different calcium responses with the amplitude of the calcium signal correlating with IFN-γ production, while IL-4 expression was induced in suboptimal conditions (40). Our findings of IL-4 production by mast cells in an oxidative microenvironment represents another manner in which these cells may contribute to the immune response. Because these cells are an important part of the innate immune response, we now establish that an oxidative microenvironment can lead to mast cell activation in vivo should unrecognized role for Fyn-dependent signaling in response to oxidative stress. These findings demonstrate a previously unrecognized role for Fyn-dependent signaling in response to oxidative stress. Studies aimed at exploring whether the oxidative microenvironment can activate mast cell responses through selective activation of Fyn-dependent signals. These findings demonstrate a previously unrecognized role for Fyn-dependent signaling in response to oxidative stress. Acknowledgment

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Disclosures

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