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Interleukin-8 Regulation of the Ras/Raf/Mitogen-activated Protein Kinase Pathway in Human Neutrophils*

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Interleukin-8 (IL-8), the prototypic member of the CXC subfamily of chemokines, induces in neutrophils chemotaxis, the respiratory burst, granule release, and increased cell adhesion. The IL-8 receptor is a seven-transmembrane spanning receptor coupled to specific heterotrimeric G proteins including Gi and G16. IL-8 stimulation of its receptor on neutrophils activates Ras GTP loading and the mitogen-activated protein kinase (MAPK) pathway including Raf-1 and B-Raf. The properties of IL-8 stimulation of the MAPK pathway differ from those observed for chemoattractants such as C5a. Even though Ras GTP loading is similar for IL-8 and C5a, the maximal activation of Raf-1 and B-Raf is approximately 2-fold and 3-7-fold, respectively, less for IL-8 than that observed for C5a. Raf-1 activation is rapid but transient, returning to near basal levels by 10 min. B-Raf activation is slower in onset and does not return to basal levels for nearly 30 min. IL-8 activation of MAPK follows a time course suggesting an involvement of both Raf-1 and B-Raf. Surprisingly, wortmannin, at low concentrations, inhibits Raf-1, B-Raf, and MAPK activation in response to IL-8 and C5a demonstrating a role for phosphatidylinositol 3-kinase in the activation of Raf kinases in G protein-coupled receptor systems in human neutrophils. Furthermore, wortmannin inhibits IL-8 stimulated granule release and neutrophil adherence. These findings demonstrate the control of Raf kinases, the MAPK pathway and specific neutrophil functions by phosphatidylinositol 3-kinase enzymes.

IL-8 belongs to a family of chemoattractant cytokines (chemokines), which is defined by a conserved protein structure (1, 2). The α subfamily of which IL-8 is the prototype consists of chemokines whose first two cysteine residues are separated by a single intervening amino acid, hence the name CXC chemokines. Members of this subfamily are clustered on human chromosome 4. The β subfamily, represented by the macrophage chemotactic protein 1, has no intervening amino acid between the first two cysteines, hence the name CC chemokines. Members of this family are located in a cluster on human chromosome 17.

IL-8 was originally identified as an activity that chemotacts neutrophils but not monocytes (3–5). Injection of IL-8 into discrete sites in the body only leads to neutrophil recruitment into these sites (6, 7). This migratory process correlates with oscillations in shape (8, 9) and filamentous actin content (10) in the neutrophils, suggesting extension and contraction of the cell as it moves toward the source of IL-8. In addition to its chemotactic function, IL-8 also triggers increased expression of CR1 (11) and CD11/CD18 complexes (12, 13), which allows for increased adhesion of neutrophils to C3b-coated particles and endothelial cells, fibrinogen, and lipopolysaccharide, respectively. Furthermore, IL-8 binding to neutrophils rapidly triggers the respiratory burst and the release of granules containing hydrolytic enzymes (14, 15), both of which contribute to the destructive properties of neutrophils. The cell functions regulated by IL-8 are the same as those induced by chemoattractants, such as C5a and fMLP, but the magnitude of the responses, especially the respiratory burst (15–17) and granule exocytosis (16), triggered by IL-8 are significantly lower (2–8-fold) than those stimulated by either C5a or fMLP.

Although the cellular functions induced by IL-8 are well characterized, the signal transduction pathways induced by IL-8 that trigger these activities have remained ill defined. IL-8 does induce the mobilization of intracellular calcium in neutrophils (15, 16). Furthermore, Wu et al. (18) have shown that IL-8 can activate phospholipase C-β, giving rise to the calcium mobilization. The activation pathways triggered beyond the level of calcium mobilization, however, remain undefined.

It is known that IL-8 initiates its effects by binding to specific receptors expressed on the surface of neutrophils. From binding studies, there are approximately 20,000–75,000 IL-8 receptors per neutrophil with a dissociation constant in the range of 0.8–4 nM (19–21). These receptors were recently cloned (22, 23) and are members of the STMR family, which couples to heterotrimeric G proteins. IL-8RA and IL-8RB share 77% homology with each other and 23–25% homology with receptors for fMLP and C5a, classic chemoattractants, which are also G protein-coupled STMRs (24). Like other chemoattractant receptors, IL-8 receptors are relatively small STMRs due to a very short cytoplasmic loop 3, making them some of the smallest STMRs known (24). Thus, the chemoattractant receptors represent a unique subfamily of STMRs. This uniqueness may be reflected
in their coupling to G proteins since loop 3 plays a critical role in determining the specificity of the G protein interaction with STMRs (25).

The two IL-8 receptors can couple to different G-proteins including members of the Goα family, Goαq, and Goαq0 and members of the Goαi family, Goαι and Goαg (18). Neutrophil responses induced by chemoattractants are partially inhibited by pertussis toxin treatment, which specifically blocks the coupling of STMRs to Gi proteins (26). The coupling of IL-8 receptors to Goαq may represent a tissue-specific interaction, since Goαq is only expressed in hematopoietic lineage cells (27). This limited tissue distribution could give rise to the activation of distinct signal transduction pathways in these cells. In addition to the α subunit coupling specificities, Wu et al. (18) found that IL-8 receptors activated phospholipase C-β2 better if expressed with Gβ2 and Gγ2 relative to other β and γ subunit combinations. Phospholipase C-β2 can be activated by free βγ subunits, which are released upon receptor activation of the heterotrimeric G protein (28). Therefore, the coupling pattern of βγ subunits might also affect the signal transduction pathways activated.

We have undertaken to define the signal transduction pathways activated by IL-8 in human neutrophils as compared with those stimulated in response to C5a and FMLP, which were previously characterized in our laboratory (29, 30). Stimulation of the C5a (29) and FMLP (30) receptors by their respective ligands triggers the MAPK pathway. Our data indicate that IL-8, like C5a and FMLP, activates the MAPK pathway through Ras/Raf-mediated events. However, the kinetics and magnitude of IL-8 regulation of the MAPK pathway are distinct from that of C5a. Additionally, our data demonstrate a requirement for PI3K in IL-8- and C5a-mediated activation of this pathway.

MATERIALS AND METHODS

Chemotactants—Recombinant, human IL-8, the 72-amino acid form, was purchased from Genzyme Corp. Recombinant, human C5a was purchased from Sigma. Several different lots of IL-8 and C5a were used with similar results. Lyophilized powders were rehydrated in lipopolysaccharide-free Krebs-Ringer phosphate buffer (pH 7.2) containing 0.5 mM NaCl, 10 mM Hepes, 1 mM EGTA, 193 mM sodium chloride, 1 mM ATP, 10 mM Hepes, 1 mM EDTA, 193 mM CaCl₂, 1 mM PMSF, 0.01 units/ml aprotinin, and 0.25 mM sodium vanadate. Cells were washed with MAPK buffer and eluted with 1 ml of MAPK buffer containing 0.5 mM NaCl. The MAPK activity in 10 μl of each eluate was assayed in triplicate in an in vitro kinase reaction using the MAPK EGFR(2Da-en)-Synthetic peptide as substrate. This size aliquot was in the linear range of the MAPK assay (data not shown). Peptide-incorporated radioactive phosphate was determined by liquid scintillation counting. 25 mM IL-8 and 50 mM C5a gave maximal activation of both MAPK and Raf kinases.

Raf Assay—The assay to measure Raf-1 and B-Raf activity was performed as described previously (34). Briefly, 4 x 10⁶ freshly isolated neutrophils were stimulated with the indicated chemoattractant for various times at 37°C. Cells were rapidly centrifuged just prior to the end of the incubation period, and lysed in 0.4 ml of 150 mM NaCl, 6 mM MgCl₂, 0.5% Triton X-100, 5 μg/ml leupeptin, and 0.09 units/ml aprotinin. The cell lysates were centrifuged for 10 min at 10,000 rpm in a refrigerated microcentrifuge to remove debris. The cleared lysates were applied to 0.5-ml DEAE-Sepharose (Pharmacia Biotech Inc.) columns that had been equilibrated with MAPK buffer. The columns were washed with MAPK buffer and eluted with 1 ml of MAPK buffer containing 0.5 mM NaCl. The MAPK activity in 10 μl of each eluate was assayed in triplicate in an in vitro kinase reaction using the MAPK EGFR(2Da-en)-Synthetic peptide as substrate. This size aliquot was in the linear range of the MAPK assay (data not shown). Peptide-incorporated radioactive phosphate was determined by liquid scintillation counting. 25 mM IL-8 and 50 mM C5a gave maximal activation of both MAPK and Raf kinases.

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Fig. 1. Stimulation of Ras GTP exchange in human neutrophils by IL-8, C5a or 12-0-tetradecanoylphorbol-13-acetate.

RESULTS

IL-8 Stimulation of Ras/GTP Exchange in Human Neutrophils—Although IL-8, C5a, and fMLP trigger similar cellular effector functions, including chemotaxis, superoxide production, and granule release, and their receptor numbers on neutrophils are similar (37), the magnitude of these responses stimulated by IL-8 is significantly lower (2–8-fold) (15–17), compared to neutrophils stimulated by C5a. Both the IL-8 (18) and C5a (38) receptors can couple to the Gt family of heterotrimeric G proteins, and previous studies have shown that Gt-coupled receptors can activate the guanine nucleotide exchange activity of Ras (39, 40). As shown in Fig. 1, Ras GTP loading was stimulated in human neutrophils in response to IL-8. The magnitude of Ras GTP loading was not significantly different between IL-8 and C5a.

IL-8 Treatment of Human Neutrophils Stimulates the MAPK Pathway Differently Than C5a—We have previously shown that C5a (29) and fMLP (30) trigger the MAPK pathway upon binding their respective receptors, and others have shown that the activation of Ras is an initiating event for MAPK activation in many systems (41–43). Because IL-8 triggered Ras activation and Ras regulates the MAPK pathway, it was of interest to know whether IL-8 could activate the MAPK pathway and if this activation was altered in any way from that seen with C5a. Similar to C5a, treatment of human neutrophils with recombinant human IL-8 induced a time-dependent activation of MAPK (Fig. 2). The maximal activation of MAPK in response to IL-8 was similar among the various neutrophil populations. However, the maximal activation of MAPK in response to C5a was dramatically different, as much as 4-fold, between populations of neutrophils. The variability in levels of MAPK activation between populations of C5a-stimulated neutrophils is similar to that seen for Ras GTP loading. However, C5a and IL-8-induced statistically equal levels of Ras guanine nucleotide exchange activity, but C5a generally had greater MAPK activity, suggesting that differences in Ras activation alone cannot account for the difference in the levels of MAPK activation.

IL-8 Activation of MAPK Follows the Activation Profiles of Raf-1 and B-Raf—Because Ras GTP loading was similar while MAPK activation was different between C5a and IL-8, the activation profile of Raf kinases in response to IL-8 versus C5a was characterized. Raf-1 and B-Raf are upstream regulators of MEK, which activates MAPK (44–46). Furthermore, both Raf-1 and B-Raf are regulated in a Ras-dependent manner (47–51). Raf-1 in human neutrophils was activated in a dose-dependent manner by recombinant human IL-8 (Fig. 3, A and B) with maximal activation occurring at 25 nM IL-8. This concentration of IL-8 gave greater than a 3-fold activation of Raf-1 relative to Raf-1 isolated from unstimulated neutrophils (Fig. 3B). The IL-8-induced activation of Raf-1 was time-dependent with a biphasic activation profile (Fig. 4, A and B). Like C5a (29), IL-8 stimulated Raf-1 and MAPK with nearly superimposable kinetics (Fig. 4, A and B, and Fig. 2). Consistent with the MAPK results, C5a activated Raf-1 to more than double the magnitude of IL-8 (Fig. 4A). IL-8 (Fig. 4, C and D) and C5a (29) stimulated similar activation profiles for B-Raf. Both chemotactic signals showed a slower onset with a prolonged activation of B-Raf versus Raf-1. B-Raf was still activated nearly 3-fold over...
background 10 min following stimulation of the neutrophils by IL-8 (Fig. 4, C and D). However, the maximal activation of B-Raf by IL-8 was approximately 3–7-fold less than that stimulated by C5a (Fig. 4C). Therefore, the regulation of Raf kinases by IL-8 is distinct from that of C5a.

Wortmannin Inhibits MAPK Activation Induced by IL-8 and C5a—Previous studies (52, 53) investigating the effects of wortmannin, a PI3K specific inhibitor at concentrations of 30–100 nM (54) on neutrophil functions did not address the mechanism by which activated PI3K and/or its products contribute to neutrophil activation. We therefore tested the effect of wortmannin on IL-8- and C5a-stimulated activation of MAPK to determine if PI3K activity could play a role in the activation of the MAPK pathway. Treating human neutrophils with wortmannin for 10 min prior to chemoattractant stimulation inhibited in a dose-dependent manner both IL-8 and C5a stimulated activation of MAPK (Fig. 5A). The ID₅₀ of wortmannin for chemoattractant induced MAPK activity was 60 nM for IL-8 and 80 nM for C5a. These values are consistent with previous reports for the inhibition of PI3K activity (54–56). The inhibition seen in the MAPK assay was not directed at MAPK specifically since addition of the inhibitor directly to the in vitro kinase reaction had no effect on the enzymatic activity of MAPK (data not shown). These data suggest that PI3K activity is required for the activation of the MAPK pathway in response to G protein-coupled receptors and in particular receptors that bind chemoattractants such as C5a and IL-8.

Wortmannin Inhibits Raf But Not Ras Activation Triggered by IL-8 and C5a—Because wortmannin inhibited the activation of MAPK in response to C5a and IL-8, it was important to know where PI3K or its products contributed to the activation of the MAPK pathway. Recently it was shown that Ras GTP could directly interact with the catalytic subunit of PI3K and that cells expressing constitutively active Ras had greatly increased levels of PI3K products (57). Therefore, we focused our attention at the level of Ras and Raf in the MAPK pathway. As one might have expected from the prediction that PI3K is downstream of Ras, wortmannin had no effect on Ras guanine nucleotide exchange activity in neutrophils stimulated with either IL-8 or C5a (data not shown). Therefore, the PI3K activity induced by either IL-8 or C5a is either downstream of Ras or activated independent of Ras.

**Fig. 3. Dose response of IL-8 stimulation of Raf-1 from human neutrophils.** Freshly isolated human neutrophils were stimulated with the indicated concentrations of IL-8 for 3 min or 50 nM C5a for 5 min at 37 °C. Cells were then lysed in 1% Nonidet P-40 and Raf-1 immunoprecipitated using a polyclonal rabbit anti-Raf-1 antiserum. Raf-1 kinase activity was measured in an in vitro kinase reaction using KM-MEK-1 as substrate. Recombinant wild-type MEK-1 was used as a control. Phosphorylated KM-MEK-1 and MEK-1 were resolved by SDS-polyacrylamide gel electrophoresis (10% polyacrylamide gel), visualized by autoradiography (A) and quantified by PhosphorImager analysis (B).

**Fig. 4. Time course of Raf-1 and B-Raf activation stimulated by IL-8 treatment of human neutrophils.** Assays were performed as described in the legend to Fig. 3 using a polyclonal rabbit anti-Raf-1 antiserum or polyclonal rabbit anti-B-Raf antiserum. Autoradiographs are of Raf-1 (A) and B-Raf (C) activity. PhosphorImager analyses are of Raf-1 (B) and B-Raf (D) activity.
Whether PI3K activation stimulated by IL-8 or C5a was downstream or independent of Ras did not change the possibility that PI3K activity might be feeding into the MAPK pathway at the level of Raf activation. As shown in Fig. 5B, wortmannin did in fact inhibit the activation of both Raf-1 and B-Raf in response to IL-8 or C5a. The ID$_{50}$ of wortmannin for IL-8-induced Raf-1 activation was less than 5 nM, while it was 7.5 nM for C5a activation of Raf-1 (data not shown). Like MAPK, the inhibition seen in the Raf assays was not directed at Raf since addition of the inhibitor directly to the in vitro kinase reaction had no effect on the enzymatic activity of Raf-1 (data not shown). Furthermore, the doses of wortmannin that inhibited MAPK and Raf activation were also effective at inhibiting the activity of PI3K isolated from human neutrophils stimulated by IL-8 (Fig. 5C). The ID$_{50}$ for Raf inhibition (<5 nM) by wortmannin was approximately 10-fold lower than that for MAPK (60 nM) in IL-8-stimulated neutrophils. This is most likely related to a modest Raf activation leading to a more significant activation of MAPK. Therefore, the data suggest that PI3K itself is involved in the regulation of Raf-1 and B-Raf activation in human neutrophils stimulated by either classic chemoattractants, such as C5a, or chemokines, such as IL-8.

The PI3K Inhibitor Wortmannin Inhibits Granule Release and Neutrophil Adherence Stimulated by IL-8—Because wortmannin inhibited the IL-8-induced activation of the MAPK pathway, we tested its action on IL-8-triggered neutrophil functions. We first tested the ability of wortmannin to inhibit IL-8-stimulated granule release from human neutrophils, as measured by the secretion of myeloperoxidase. Wortmannin inhibited a dose-dependent manner (ID$_{50}$ of 24 nM) the IL-8-induced secretion of myeloperoxidase (Fig. 6). This is consistent with a previous report in which wortmannin inhibited C5a and fMLP triggered granule release from neutrophils (53). In contrast, neutrophil adherence was less sensitive to wortmannin with a projected ID$_{50}$ of greater
than 50 nM (Fig. 6). This finding suggests that neutrophil adherence is less dependent on PI3K activity than secretion. Therefore, PI3K activity appears to regulate secretion and adherence to different degrees.

**DISCUSSION**

In the present study, we have determined the effect of the chemokine IL-8 on the activation of the Ras/Raf/MAPK pathway in human peripheral blood neutrophils. We also investigated the role of PI3K in the activation of this pathway by IL-8 and C5a. IL-8 and C5a activated the MAPK pathway. Both IL-8 and C5a activated not only Raf-1 but also B-Raf, a homolog of Raf-1 (58, 59), in neutrophils and the guanine nucleotide exchange activity of Ras. Interestingly, the levels of MAPK, Raf-1, and B-Raf activation stimulated by IL-8 versus C5a were significantly different even though receptor numbers for the two ligands are similar in human neutrophils (37). Surprisingly, the PI3K inhibitor wortmannin inhibited the IL-8- and C5a-induced activation of Raf-1 and B-Raf, resulting in the inhibition of MAPK stimulation. This represents the first demonstration of a role for PI3K in the activation of Raf proteins by G protein-coupled receptor systems in human cells.

IL-8-induced activation of the MAPK pathway was similar to but distinct from that induced by C5a. Even though IL-8-stimulated Ras activation was not significantly different from that for C5a, the activation of both Raf-1 and B-Raf was less with IL-8. Similarly, MAPK activation was greater for C5a as compared with IL-8. Surprisingly, although the maximum level of MAPK activation in response to IL-8 was similar in populations of neutrophils from different donors, it was more variable, as much as 4-fold, among the same populations of neutrophils when stimulated by C5a. This may reflect differences in the sensitivity of C5a receptor coupling among donors. Whether this is due to differences in circulating C5a levels or receptor numbers in donors or a regulatory event downstream of agonist binding is presently unclear. Nonetheless, C5a activation of the MAPK pathway is more robust than that for IL-8 in multiple donors.

Our results begin to define at a biochemical level the differential regulation of neutrophil functions in response to chemokines and classic chemotaxins. The IL-8 and C5a receptors are both believed to predominantly couple to G proteins (18, 26, 60) with similar numbers of receptors per neutrophil (37). However, C5a activates both Raf-1 and B-Raf as well as MAPK to significantly greater levels than that for IL-8. Thus, the receptors for C5a and IL-8 are differentially controlling the magnitude of neutrophil intracellular signaling. The difference in the magnitude of signaling stimulated by IL-8 and C5a may contribute to their differences in neutrophil activation.

The ability of wortmannin to inhibit the MAPK pathway in human neutrophils has now been described. This is in agreement with previous reports in which wortmannin inhibited insulin (61, 62) and platelet activating factor (63) stimulation of the MAPK pathway in rodent cells. In our system, the inhibition of PI3K by wortmannin affects the activation of both Raf-1 and B-Raf. This is in sharp contrast to a recent report by Karnitz et al. (64) in which wortmannin inhibited the MAPK pathway stimulated by IL-2 at the level of MEK but not Raf. This difference could be a reflection of a difference between tyrosine kinase-coupled versus G protein-coupled receptor systems, IL-2 versus IL-8, respectively. The IL-2 receptor regulates p85/p110 PI3K (65), while the IL-8 receptor activates both p85/p110 PI3K and PI3K-γ (66, 67). The data thus suggest that at least a subset of cell types require a PI3K activity for MAPK activation. How PI3K regulates Raf-1/B-Raf activation is presently unclear, but it might alter localized membrane properties and/or the activity of other kinases involved in Raf-1/B-Raf regulation (68). The finding that human neutrophils and some but not all rodent cell types studied have a PI3K requirement for Raf-1/B-Raf activation suggests that there is more than one mechanism to control Raf activation. Neutrophils provide a robust response to IL-8 and C5a to define the PI3K-dependent pathway in future studies.

Finally, wortmannin more potently inhibits the respiratory burst (52, 53) and granule secretion (53) compared with neutrophil adherence, suggesting that specific signaling pathways involving PI3K differentially control these responses. Our findings that PI3K is involved in the regulation of the MAPK pathway indicates that in human neutrophils, the control of phospholipase C-β, PI3K, and the MAPK pathway are highly integrated events (29, 66). This complicates the dissection of signals dominant in the control of specific neutrophil functions but also provides a mechanism where by the host defense mechanisms are coordinately regulated in the neutrophil. Biochemical and genetic manipulation of these signal components will be required to define their precise role in neutrophil functions.

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