Diverging Signal Transduction Pathways Activated by Interleukin 8 (IL-8) and Related Chemokines in Human Neutrophils

IL-8 AND Gro-α DIFFERENTIALLY STIMULATE CALCIUM INFLUX THROUGH IL-8 RECEPTORS A AND B*

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Interleukin 8 (IL-8) and Gro-α are members of the CXC branch of a family of cytokines recently designated the "chemokine" superfamily. Recent evidence indicates that, contrary to previously held beliefs, IL-8 and Gro-α may not be perceived equivalently by neutrophils. In this study, we have evaluated the effects of IL-8 and Gro-α on the rate of calcium influx in human neutrophils and in 293 cells transfected with type A or type B IL-8 receptors. Of these two chemokines, only Gro-α induced an influx of calcium in neutrophils as judged by the sensitivity of the mobilization of calcium to the extracellular calcium chelator EGTA and to the nonselective divalent cation channel inhibitor SK&F 96365, as well as by manganese quenching experiments. IL-8 was similarly without effect on the rate of Mn²⁺ influx in 293 cells transfected with IL-8 receptor A (IL-8RA) or IL-8RB. On the other hand, Gro-α induced an SK&F 96365-sensitive increase of the rate of Mn²⁺ influx in IL-8RB, but not in IL-8RA-transfected 293 cells. These results indicate not only that neutrophils respond differently to IL-8 than they do to Gro-α but, furthermore, that the consequences of the binding of IL-8 and Gro-α to IL-8RB are distinct.

An important aspect of host defense is the ability of polymorphonuclear neutrophils (neutrophils) to leave the blood stream and enter tissues. Accordingly, the locomotive ability of these cells, as well as the stimuli (chemotaxis) that affect this ability, are critical elements of their functional responsiveness.

IL-8 belongs to a family of small proteins of 8–10 kDa that exhibit chemotactic activity toward various leukocyte subsets (1–3). Alignment of the cysteine residues distinguishes between two groups of peptides within this family: one with the first two cysteines separated by one amino acid (C chemokines), and the other with the first two cysteines adjacent (CC chemokines). IL-8, NAP-2, and Gro gene products (Gro-α, Gro-β, Gro-α) belong to the first group (4, 5). In vitro, these chemokines appear to activate neutrophils in a highly selective manner (1–3).

Two specific IL-8 receptors have been identified on the surface of human neutrophils: IL-8RA and IL-8RB. IL-8RB binds IL-8, NAP-2, and Gro-α with high affinity, whereas IL-8RA shows high affinity for IL-8 but only low affinity for NAP-2 and Gro-α (6, 7). The amino acid sequences encoded by the cDNA clones of IL-8 receptors show that they belong to the superfam-ily of G-protein-linked receptors (8–11). Consistent with the last observation, a physical association between the IL-8 receptors and the α-subunit of G₅ has recently been observed in human neutrophils and in 293 cells transfected with IL-8RA or IL-8RB receptors (12). Furthermore, a functional linkage between IL-8R and phospholipase C has been observed upon the co-transfection of the receptors and the α-subunit of G₅-like G proteins (11), and most, if not all, IL-8-induced neutrophil responses are sensitive to inhibition by pertussis toxin (13).

It has generally been assumed that neutrophils respond similarly to all CXC chemokines and that the two types of IL-8 receptors are functionally interchangeable. The adequacy of this model has, however, recently been challenged. More specifically, IL-8 stimulates the activity of phospholipase D, under-agarose chemotaxis, and the up-regulation of CD11b/CD18 to a significantly greater extent than either Gro-α or NAP-2 (14).

The biochemical basis for the differences in neutrophil responses to IL-8 versus Gro-α and NAP-2 is not yet known. While co-immunoprecipitation experiments demonstrate that the divergence in the signal transduction pathways apparently lies downstream from the initial interaction between IL-8 and the α-subunit of G₅ (12), further studies are required to identify the points at which the divergence occurs. The present study was therefore initiated to further examine the signal transduction pathways elicited through the two IL-8 receptor types by taking advantage of the availability of 293 cells transfected with IL-8RA or IL-8RB and comparing the ability of IL-8 and Gro-α to stimulate an influx of calcium in neutrophils and transfected 293 cells. The results obtained indicate not only that neutrophils respond differently to IL-8 as opposed to Gro-α but, furthermore, that they possess the ability to differentiate between IL-8 and Gro-α interacting with IL-8RB.

MATERIALS AND METHODS

Reagents and Antibodies—Indo-1/AM and Fura-2/AM were obtained from Molecular Probes (Junction City, OR). Hanks’ balanced salt solution.
**Fig. 1. Effect of IL-8 and Gro-α on the mobilization of calcium in human neutrophils.** The experimental conditions are described under "Materials and Methods." The arrows indicate the time of addition of the agonists (10^{-7} M). Panel A represents the raw, untransformed data. In panel B, the curves were adjusted to have the same peak heights. The results are representative of at least three independent determinations.

**RESULTS**

**Human Neutrophils—** The addition of IL-8 or Gro-α to a suspension of human neutrophils induced a transient increase in the concentration of cytoplasmic free calcium (Fig. 1A). While the kinetics of the initial rise in cytoplasmic free calcium initiated by IL-8 and Gro-α are essentially identical, the calcium signal induced by Gro-α was consistently observed to be longer lasting than that induced by IL-8. This is evidenced in Fig. 1B, in which the signals observed in response to the agonists were normalized to the same peak heights, by the presence of a delayed shoulder in the Gro-α response, which is absent in cells stimulated by IL-8. No significant qualitative or quantitative differences were seen in the calcium signals induced by IL-8 at concentrations ranging from 10^{-7} to 10^{-5} M or by IL-8 added first or after the Gro-α signal had returned to base line, i.e., at a time when most, if not all, IL-8RB was still occupied by Gro-α (as evidenced by a lack of response to a second addition of Gro-α (data not shown)).

**Intracellular Free Calcium—** Intracellular free calcium was measured using the fluorescent probes Indo-1 or Fura-2 as described previously (16, 17). Cell suspensions (10^7 cells/ml) were loaded with Indo-1/AM (1 μM) or Fura-2/AM (1 μM, 30 min at 37°C), washed, and resuspended at 2 x 10^6 cells/ml in HBSS supplemented with 0.5 or 1.6 mM calcium as indicated, and their fluorescence was monitored in a spectrofluorometer (SLM 8000, SLM-Aminco, Champagne, IL) (excitation and emission wavelengths, 350 and 405 nm for Indo-1 and 340 and 510 for Fura-2, respectively). The internal calcium concentrations were calculated as described in Tsien et al. (18). Each run was individually calibrated. The integrated areas under the calcium mobilization curves were computed using the FigP program (Biosoft, Cambridge, United Kingdom).

**Manganese Influx—** Manganese influx was measured using the fluorescent probes Fura-2/AM or Indo-1/AM as described previously (19). The cells suspensions (10^7 cells/ml) were loaded with Fura-2/AM (1 μM) (neutrophils) or Indo-1/AM (1 μM) (293 cells) for 30 min at 37°C, washed, and resuspended at 2 x 10^6 cells/ml in HBSS without calcium. MnCl_2 (100 μM) was added 1 min before stimulation of the cells. The fluorescence of the cells was monitored in an SLM 8000 spectrofluorometer (SLM-Aminco) (excitation and emission wavelengths, 360 and 505 nm for Fura-2, and 350 and 455 nm for Indo-1).

**RESULTS**

Human Neutrophils—The addition of IL-8 or Gro-α to a suspension of human neutrophils induced a transient increase in the concentration of cytoplasmic free calcium (Fig. 1A). The calcium signal induced by Gro-α was consistently observed to be longer lasting than that induced by IL-8. This is evidenced in Fig. 1B, in which the signals observed in response to the agonists were normalized to the same peak heights, by the presence of a delayed shoulder in the Gro-α response, which is absent in cells stimulated by IL-8. No significant qualitative or quantitative differences were seen in the calcium signals induced by IL-8 at concentrations ranging from 10^{-7} to 10^{-5} M or by IL-8 added first or after the Gro-α signal had returned to base line, i.e., at a time when most, if not all, IL-8RB was still occupied by Gro-α (as evidenced by a lack of response to a second addition of Gro-α (data not shown)).

The contribution of extracellular calcium to the increase in cytoplasmic free calcium induced by IL-8 and Gro-α was then evaluated by adding the calcium chelator EGTA to the cell suspensions 30 s before stimulating the cells with IL-8 or Gro-α. Under these conditions, little, if any, extracellular calcium is available to diffuse into the cells, and the changes in fluorescence of Fura-2 that are recorded can be assumed to be almost exclusively due to calcium released from internal stores (20). The results of these experiments are summarized in Fig. 2. The tracings illustrated in panels A and B demonstrate that while the addition of EGTA had little to no effect on the response to IL-8, it significantly reduced that to Gro-α. The analysis of three such independent experiments indicated that EGTA reduced by 55% the increase in the intracellular levels of calcium induced by Gro-α, whereas it had no detectable inhibitory effect on the responses to IL-8 (Fig. 2C). It was also observed, in control experiments, that the tyrosine phosphorylation responses to IL-8 and to Gro-α were unaffected by EGTA, thereby indicating that the latter did not grossly interfere with the binding of the chemokines (data not shown).

The effect of a selective inhibitor of receptor-mediated calcium entry, SK&F 96365, on the mobilization of calcium induced by IL-8 and Gro-α was examined next. The cells were preincubated with 25 μM SK&F 96365 for 5 min and then stimulated by 10^{-7} M IL-8 or Gro-α (Fig. 3). Representative tracings are shown in panels A and B, and a statistical analysis of the results of the three experiments is shown in panel C. SK&F 96365 reduced to a small but not significant (p > 0.05) extent the magnitude of the mobilization of calcium induced by IL-8. On the other hand, SK&F 96365 had a pronounced effect on the response to Gro-α (60 ± 5% inhibition, p < 0.05).

The differences in the shape of the calcium mobilization curves induced by IL-8 and Gro-α in the effects of EGTA and SK&F 96365 on these responses suggest that the two chemokines do not affect similarly the permeability to calcium of the plasma membrane of neutrophils. This parameter can be monitored more directly at the isosbestic point of Fura-2 by following the quenching of the fluorescent probe resulting from the influx of extracellularly added manganese (19). The effects of IL-8 and of Gro-α on Mn^{2+} influx in neutrophils (as monitored by the rate quenching of Fura-2) were therefore exam-
ined, and the results of these experiments are illustrated in Fig. 4.

The addition of IL-8 induced only a very small increase in the rate of quenching of Fura-2 by Mn^{2+}. On the other hand, Gro-α transiently, and significantly, stimulated the rate of decrease in the fluorescence of Fura-2, indicating that it had enhanced the rate of entry of Mn^{2+} into the cells. The binding of IL-8 to its two receptors was verified by demonstrating that Gro-α was inactive when added after IL-8. NAP-2 (100 nM) also increased the rate of Fura-2 quenching in the presence of MnCl_2 (data not shown). Furthermore, the entry of Mn^{2+} inside the cells induced by Gro-α was completely blocked by the addition of 25 μM SK&F 96365 (data not shown).

Transfected 293 Cells—The cDNAs coding for IL-8RA and IL-8RB have been stably expressed in a human embryonic kidney cell line (15). These transfecants express large numbers of IL-8 receptors (about 500,000/cell) that display binding affinities similar to those of the native neutrophil receptors (K_d = 2 nM). The ability of IL-8 and Gro-α to induce a pertussis toxin-sensitive mobilization of calcium in the expected transfecants (293 IL-8RA and IL-8RB for IL-8 and 293 IL-8RB for Gro-α) has also been demonstrated (12). The availability of these cell lines allows the direct testing of the responses to each receptor type, a possibility that neutrophils do not permit, since they express both receptors. In this series of experiments, 293 cells transfected with IL-8RA or IL-8RB were loaded with Indo-1/AM and stimulated with the indicated chemokines in the presence of Mn^{2+}, and the rates of Indo-1 fluorescence quenching in response to IL-8 and Gro-α were monitored.

Gro-α stimulated a rapid and significant decrease in Indo-1 fluorescence in 293 IL-8RB cells (Fig. 5A). IL-8 was without effect on its own in these cells, although its addition was able to inhibit the subsequent response to Gro-α (Fig. 5A), an inhibition that is likely due either to the occupation of the receptors by the saturating concentrations of the chemokines used in this assay (10^{-7} M) or to presently undefined desensitization mechanisms. Neither of the two chemokines induced significant changes in the rates of Mn^{2+} influx in 293 IL-8RA cells (Fig. 5B). A statistical analysis of the results of three such independent determinations confirmed these observations (Fig. 5B). The increased rate of Indo-1 quenching induced by Gro-α was inhibited by more than 95% by SK&F 96365 (data not shown).

DISCUSSION

The present study was undertaken to examine the signal transduction pathways activated in human neutrophils and IL-8R-transfected 293 cells upon the activation of each of the two IL-8 receptor subtypes. Previous data indicate that the signal transduction pathways through IL-8RA and RB diverge in vitro chemotaxis (as determined by the under-agarose method), and exocytosis (enhanced surface expression of CD11b/CD18).
Although the binding affinities of IL-8 and Gro-a toward IL-8RA and IL-8RB are known (6, 8) and some of the functional responses of neutrophils to these two chemokines have been identified (6, 21–24), the specific transduction pathways linked to the two IL-8R subtypes have only partially been examined. The results of this investigation have led to the novel observation that the two CXC chemokines activate distinct intracellular effector systems pertaining to the mobilization of calcium upon the ligation of IL-8RB.

The modulation of the levels of free cytoplasmic free calcium represents one of the major signaling systems in mammalian cells (25). Upon appropriate stimulation, calcium is derived from intracellular and extracellular sources by separate, although likely interdependent, mechanisms (25). In the present study, several lines of evidence indicate that IL-8 and Gro-a rely to different extents on these two sources of calcium and, more specifically, that only Gro-a induces a significant influx of calcium in human neutrophils. The most direct confirmation of the differential effects of IL-8 and Gro-a on calcium influx in neutrophils was derived from the Mn^{2+} quenching experiments, which established that only Gro-a caused the opening of plasma membrane divalent cation channels. These data are in accordance with the differences in the shape of the calcium mobilization curves generated in response to IL-8 and Gro-a and with the differential sensitivity of intracellular calcium mobilization induced by IL-8 or Gro-a to EGTA and to the divalent cation channel inhibitor SK&F 96365.

Previous studies indicate that IL-8 is significantly more potent than Gro-a in inducing activation of phospholipase D, up-regulation of surface expression of CD11b/CD18, and in vitro chemotaxis (14). The present results extend these observations to the level of second messenger generation. It is unclear, however, whether a causal relationship exists between the opening of divalent cation channels and the functional
responses previously monitored (i.e. phospholipase D, CD11b/CD18 expression, chemotaxis).

The differences in the characteristics of the calcium responses of human neutrophils to IL-8 and Gro-α can be explained by postulating that the two receptor subtypes are coupled to distinct effector systems (i.e. only IL-8RB being linked to an increase in plasma permeability) or that the functional consequences of the occupation of IL-8RB by IL-8 or Gro-α are not equivalent. Since neutrophils express both receptor types, it is not possible to distinguish between these two hypotheses in this cellular system. Therefore, these alternative explanations were directly tested in 293 cells transfected with either IL-8RA or IL-8RB. The results of the experiments conducted with 293 cells provided significant support for the second alternative. The first hypothesis, i.e. that the two receptor subtypes are coupled to distinct effector systems, was effectively eliminated by the observation that IL-8 failed to stimulate a significant influx of calcium irrespective of whether it was bound to IL-8RA or IL-8RB. This result is in accord with the lack of effect of IL-8 on Mn^{2+} influx in neutrophils, even though IL-8 binds equally well to both receptor types. In contrast, Gro-α stimulated the influx of calcium in 293 IL-8RB cells as well as in neutrophils. This directly supports the second hypothesis. Thus, the data obtained with the transfected 293 cells not only corroborate those acquired in neutrophils, but they also provide strong evidence that the consequences of the occupation of IL-8RB depends on the agonist to which the cells are exposed. The mechanism(s) by which the cells discriminate between these two agonists that exhibit similar binding affinities toward this receptor (6, 8) are unknown at present. They may be dependent, in part at least, on differences in the kinetics of binding of CXC chemokines to IL-8RB. In addition, it should be pointed out that while the molecular determinants of the binding of IL-8 to IL-8RA have been elucidated (26), only partial data are available with IL-8RB or with Gro-α. The latter indicate that IL-8, NAP-2, and Gro-α bind to distinct, although overlapping, sites on IL-8RB (27). These data raise the possibility that these ligands induce different conformational changes in IL-8RB that lead to the differences in the responses observed in the present study.

These results raise several intriguing questions that range from basic cell physiology to the biology of the CXC chemokines. The data obtained, although not derived from experiments designed to directly test this particular point, are clearly not well described by the capacitative model of calcium entry (25). IL-8 and Gro-α mobilize intracellular calcium to essentially identical extents, but only the latter stimulates calcium entry into the cells. The resolution of this apparent paradox is likely to prove difficult in view of our presently limited understanding of the mechanisms underlying the regulation of the plasma membrane divalent cation channels and of those concerning the interaction of chemokines with their receptors.

Finally, the present data raise the other intriguing possibility that IL-8 and Gro-α are not functionally redundant chemokines but that they may possess distinct spectra of biological activities toward neutrophils and/or other inflammatory or immune cells. It is of particular interest that IL-8RB is expressed on a wider range of cell types including cells outside the immune system (28) and has been shown to mediate effects not associated with other chemokines. Such effects include the modulation of melanocyte growth (29) and of collagen synthesis in synovial fibroblasts (30).

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