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Clustering of Urokinase Receptors (uPAR; CD87) Induces Proinflammatory Signaling in Human Polymorphonuclear Neutrophils


Leukocytes use urokinase receptors (uPAR; CD87) in adhesion, migration, and proteolysis of matrix proteins. Typically, uPAR clusters at cell-substratum interfaces, at focal adhesions, and at the leading edges of migrating cells. This study was undertaken to determine whether uPAR clustering mediates activation signaling in human polymorphonuclear neutrophils. Cells were labeled with fluo-3/AM to quantitate intracellular $[\text{Ca}^{2+}]_i$ by spectrofluorometry, and uPAR was aggregated by Ab cross-linking. Aggregating uPAR induced a highly reproducible increase in $[\text{Ca}^{2+}]_i$ (baseline to peak) of 295 ± 37 nM ($p = 0.0002$). Acutely treating cells with high m.w. urokinase (HMW-uPA; 4000 IU/ml) produced a response of similar magnitude but far shorter duration. Selectively aggregating uPA-occupied uPAR produced smaller increases in $[\text{Ca}^{2+}]_i$, but saturating uPAR with HMW-uPA increased the response to approximate that of uPAR cross-linking. Cross-linking uPAR induced rapid and significant increases in membrane expression of CD11b and increased degranulation (release of $\beta$-glucuronidase and lactoferrin) to a significantly greater degree than cross-linking control Abs. The magnitude of degranulation correlated closely with the difference between baseline and peak $[\text{Ca}^{2+}]_i$, but was not dependent on the state of uPA occupancy. By contrast, selectively cross-linking uPA-occupied uPAR was capable of directly inducing superoxide release as well as enhancing FMLP-stimulated superoxide release. These results could not be duplicated by preferentially cross-linking unoccupied uPAR. We conclude that uPAR aggregation initiates activation signaling in polymorphonuclear neutrophils through at least two distinct uPA-dependent and uPA-independent pathways, increasing their proinflammatory potency (degranulation and oxidant release) and altering expression of CD11b/CD18 to favor a firmly adherent phenotype. The Journal of Immunology, 2000, 165: 3341–3349.

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1 Abbreviations used in this paper: PMN, polymorphonuclear neutrophil; uPAR, urokinase plasminogen activator receptor; $[\text{Ca}^{2+}]_i$, intracellular $\text{Ca}^{2+}$ concentration; uPA, urokinase plasminogen activator; $\Delta [\text{Ca}^{2+}]_i$, difference between baseline and peak $[\text{Ca}^{2+}]_i$; CR3, complement receptor 3; HMW-uPA, high m.w. urokinase plasminogen activator.
trisphosphate, followed by increases in the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) that were blocked by 1) depleting intracellular Ca\(^{2+}\) stores with thapsigargin, 2) inhibiting phospholipase C with U73122, and 3) inhibiting tyrosine kinase with herbimycin A, but were less affected by reducing extracellular Ca\(^{2+}\) (27). From these findings, it was concluded that uPAR aggregation triggers phosphoinositide hydrolysis and mobilization of intracellular Ca\(^{2+}\), with secondary influxes of extracellular Ca\(^{2+}\). Recognizing that human PMN express uPAR both on the plasma membrane and in a readily mobilized pool in intracellular granules, we sought in this study to determine whether uPAR aggregation can also initiate activation signaling in human PMN and regulate the proinflammatory phenotype and function of these cells.

Materials and Methods

Reagents

Purified murine IgG Fc fragments and F(ab\(^\prime\))\(_2\) of goat Ab reactive against murine IgG F(ab\(^\prime\))\(_2\) were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Anti-uPAR mAb (IgG2a; clone 3B10) was purified from mouse ascites by protein A-Sepharose and quantitated by protein content (29). The murine anti-uPAR mAb recognizes an epitope near the uPA binding site and thus preferentially binds to unoccupied uPAR, as shown by immunofluorescence microscopy that virtually identical protocols for Ab-mediated uPAR cross-linking in human neutrophils produce readily evident receptor capping in approximately one-half of labeled cells (10, 22). To selectively aggregate uPA/uPAR complexes, cells were blocked with Fc fragments as described above and then treated with an anti-uPA mAb (100 \(\mu\)g/ml; 3940A, American Diagnostica) or an IgG1 control, followed by the F(ab\(^\prime\))\(_2\) of goat anti-mouse F(ab\(^\prime\))\(_2\) (100 \(\mu\)g/ml) as described above. In some instances, available uPA were preloaded with an excess of HMW-uPA (1 \(\mu\)g/ml, -20 nM) for 15 min at 4°C in experimental buffer, washed, and then subjected to cross-linking with the anti-uPA mAb. To confirm that HMW-uPA added in this way binds predominately to uPAR, PMNs were pretreated with a polyclonal rabbit anti-human uPA Ab or control rabbit IgG, followed by FITC-labeled uPA (1 \(\mu\)g/ml for 15 min, 4°C; American Diagnostica). Flow cytometry confirmed that the anti-uPA Ab inhibited binding of the FITC-uPA by 93 ± 3.7% relative to the control Ab (mean ± SEM; \(n = 3\)).

Measurement of [Ca\(^{2+}\)]\(_i\)

Cells were loaded (5 \(\times\) \(10^6\)/ml) with the Ca\(^{2+}\)-sensitive fluorescent dye fluo-3/AM (2 \(\mu\)M; Molecular Probes, Eugene OR) at 30°C for 30 min in 145 mM NaCl/5 mM KCl/1 mM MgCl\(_2\)/10 mM glucose/1 mM CaCl\(_2\)/1% (w/v) BSA/10 mM HEPES, pH 7.4. Cells were then incubated with murine IgG Fc fragments (150 \(\mu\)g/ml) at 4°C for 15 min to block binding of the primary Abs to Fc receptors. Cells were then incubated with the anti-uPA mAb or a control IgG2a mAb (100 \(\mu\)g/ml) at 4°C for 30 min, and washed in experimental buffer at 4°C. To initiate receptor cross-linking, F(ab\(^\prime\))\(_2\) of goat anti-mouse F(ab\(^\prime\))\(_2\) Ab (100 \(\mu\)g/ml) were added after warming the cells to 37°C. Previous studies have shown by immunofluorescence microscopy that virtually identical protocols for human PMN cross-linking in human neutrophils produce readily evident receptor capping in approximately one-half of labeled cells (10, 22). To selectively aggregate uPA/uPAR complexes, cells were blocked with Fc fragments as described above and then treated with an anti-uPA mAb (100 \(\mu\)g/ml; 3940A, American Diagnostica) or an IgG1 control, followed by the F(ab\(^\prime\))\(_2\) of goat anti-mouse F(ab\(^\prime\))\(_2\) Ab (100 \(\mu\)g/ml) as described above. In some instances, available uPA were preloaded with an excess of HMW-uPA (1 \(\mu\)g/ml, -20 nM) for 15 min at 4°C in experimental buffer, washed, and then subjected to cross-linking with the anti-uPA mAb. To confirm that HMW-uPA added in this way binds predominately to uPAR, PMNs were pretreated with a polyclonal rabbit anti-human uPA Ab or control rabbit IgG, followed by FITC-labeled uPA (1 \(\mu\)g/ml for 15 min, 4°C; American Diagnostica). Flow cytometry confirmed that the anti-uPA Ab inhibited binding of the FITC-uPA by 93 ± 3.7% relative to the control Ab (mean ± SEM; \(n = 3\)).

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Neutrophil degranulation

\(\beta\)-Glucuronidase (a marker of azurophilic granules) was measured by incubating conditioned medium 1:4 (v/v) with 10% phenolophosphate \(\beta\)-mono-glucuronate in 70 mM sodium acetate buffer, pH 4.5, for 18 h at 37°C. The reaction was then stopped with 0.4 M glycine buffer, pH 10.5, and read in a plate spectrophotometer at 405 nm. The reaction product was standardized against known quantities of phenolophosphate, and release was expressed as a percentage of the total cellular content in excess of unstimulated cells cultured in parallel (33). Lactoferrin (a marker for specific granules) was assayed by ELISA, using a rabbit anti-human lactoferrin capture IgG and a peroxidase-conjugated rabbit anti-human lactoferrin detection IgG (ICN/Cappel, Costa Mesa, CA), developed with a standard peroxidase diaminochrome substrate and standardized to purified human lactoferrin (34, 35). Data release are expressed as nanograms per millilitre released in excess of unstimulated control cells.

Superoxide release

PMNs were incubated in 145 mM NaCl/5 mM KCl/1 mM MgCl\(_2\)/10 mM glucose/1 mM CaCl\(_2\)/1% (v/v) BSA/10 mM HEPES, pH 7.4, with Fe\(^{3+}\) cytochrome c (675 \(\mu\)g/ml; Sigma, St. Louis, MO) with or without superoxide dismutase (250 \(\mu\)g/ml; Sigma) for 45 min at 37°C. The reaction was terminated by rapid chilling to 4°C and removing the cells by centrifugation. Conditioned medium was then transferred to microtitre wells and read at 550 nm in a plate spectrophotometer. Superoxide production was determined from a standard curve relating absorbances to known quantities of sodium dithionite-reduced Fe\(^{3+}\) cytochrome c, and data are expressed as nanomoles of superoxide released per 45 min/10\(^6\) cells (36).

Statistical analysis

Individual comparisons of group means were performed with two-tailed Student’s \(t\) tests, with \(p \leq 0.05\) considered significant. Multiple comparisons were performed using one-way ANOVA, with Dunnett’s post-test for multiple comparisons to a single control or Bonferroni’s post-test for multiple selected comparisons, as indicated. All analyses were performed with GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA).

Results

Expression of uPAR by human PMNs

uPAR was demonstrable by flow cytometry on 34 ± 3.6% (mean ± SEM; \(n = 9\)) of PMNs immediately after purification, in keeping with prior observations that uPAR is expressed on PMNs at relatively low levels before stimulation with proinflammatory agents (25). There was significant interdonor variability in the quantity of uPAR expressed, with the sampled population expressing 2.3 ± 1.24 arbitrary units (mean ± SD; coefficient of variation, 54%; range, 0.82–4.3). Flow cytometry was also used to determine the occupancy state of uPAR by measuring uPA on the cell surface relative to the quantity of uPA bound after prior treatment with HMW-uPA (1 \(\mu\)g/ml for 15 min at 4°C). Immediately upon purifying the PMNs, 30.3 ± 4.3% (mean ± SEM; \(n = 9\)) of available uPA binding sites were occupied with uPA.

Immuno fluorescence flow cytometry

For immunolabeling, cells were resuspended in labeling buffer (PBS with 0.1% human \(\gamma\)-globulin and 0.1% glucose, pH 7.4) and incubated with the specified primary mAb for 30 min, 4°C, followed by PE-conjugated goat anti-mouse Ab (30 min, 4°C). For negative controls, cells were stained with secondary mAb alone and with an irrelevant isotype-matched primary Ab. In some instances FITC-conjugated primary Abs were used along with an FITC-labeled isotype-matched Ab as a control. Fluorescence intensity was recorded as a measure of relative Ag expression using EPICS Elite ESP flow cytometer (Coulter, Miami, FL; University of Michigan Flow Cytometry Core Facility). Gating was determined by forward and 90° light scatter characteristics. Mean fluorescence intensities (linear scale) were determined from \(\geq\)10,000 cells, and specific fluorescence intensities were calculated by subtracting the corresponding value of the nonspecific control. To maintain consistent results between experiments, the flow cytometer was adjusted to provide constant fluorescence intensities for Coulter Standard Brite beads.

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Effect of uPAR aggregation on intracellular Ca$^{2+}$ concentrations

Ab-mediated cross-linking of uPAR was performed to determine whether uPAR aggregation stimulates a change in [Ca$^{2+}$]$_{i}$. Cross-linking uPAR increased [Ca$^{2+}$]$_{i}$ from a baseline of 207 ± 9.6 nM to a peak of 501 ± 41.7 nM (p < 0.0001). The difference between baseline and peak [Ca$^{2+}$]$_{i}$ (Δ[Ca$^{2+}$]$_{i}$) was 295 ± 37 nM (Fig. 1). By contrast, cross-linking an isotype-matched control mAb did not significantly affect [Ca$^{2+}$]$_{i}$ (baseline and peak [Ca$^{2+}$]$_{i}$, 188 ± 9.8 and 188 ± 10.4 nM, respectively; n = 9). Likewise, neither the primary mAb nor cross-linking Ab alone affected [Ca$^{2+}$]$_{i}$ (not shown). The uPAR has a single binding site for uPA, and uPA is monovalent toward uPAR (6), so it is expected that uPA/uPAR complexes have no direct mechanism for aggregation without a cross-linking agent, in this case the secondary Ab. The increase in [Ca$^{2+}$]$_{i}$ in response to uPAR aggregation began 38 ± 4.8 s after introducing the cross-linking Ab, consistent with a response contingent upon receptor redistribution. The duration of the response was 127 ± 6.2 s. By comparison, the Δ[Ca$^{2+}$]$_{i}$ was 2- to 3-fold greater in PMNs than previously observed for U937 cells and freshly purified human monocytes, while all cell types yielded [Ca$^{2+}$]$_{i}$ responses of very similar duration (27). To provide another frame of reference for PMNs, fluo-3/AM-labeled PMNs were stimulated with FMLP (5 × 10$^{-7}$ M), producing virtually immediate, large, and transient
increases in [Ca\^2\textsuperscript{+}], (Fig. 2). This result is consistent with a synchronous response to a simple ligand-receptor interaction, whereas uPAR aggregation probably occurs asynchronously among the cells, yielding a [Ca\^2\textsuperscript{+}] transient of longer duration and a lower apparent peak. To further compare the [Ca\^2\textsuperscript{+}] responses, the areas under the respective curves were quantitated to measure the increases in [Ca\^2\textsuperscript{+}] integrated over time. In this respect, the magnitude of the [Ca\^2\textsuperscript{+}] response to uPAR aggregation compared more favorably with FMLP, producing approximately one-half the integrated Ca\^2\textsuperscript{+} signal (Fig. 2B).

PMNs were also treated with high concentrations of HMW-uPA (4000 IU/ml, ~44 \textmu M) to compare the effects on Ca\^2\textsuperscript{+} signaling of uPAR ligation vs uPAR aggregation. Similar to findings reported previously (22), binding uPA to uPAR elicited increases in [Ca\^2\textsuperscript{+}], (Fig. 3). However, the response differed from that elicited by uPAR aggregation in several important respects. First, like FMLP, the onset of the response to HMW-uPA was virtually immediate, consistent with a simple ligand-receptor interaction. Secondly, the response was relatively brief. As shown by a representative tracing in Fig. 3A, there was an initial spike of 10 s or less, followed by a gradual return to baseline. The overall duration of the response was 63.3 \pm 4.1 s, which was one-half the duration of the [Ca\^2\textsuperscript{+}], response to uPAR aggregation. The \Delta [Ca\^2\textsuperscript{+}], integrated over time indicates that the magnitude of the Ca\^2\textsuperscript{+} signal to uPAR cross-linking exceeded the response to HMW-uPA ligation by >3-fold (Fig. 3B). Stimulation with 2000 IU/ml HMW-uPA, although still far exceeding the 0.1–0.5 nM \textit{K_d} reported for uPAR (6), did not have a significant effect on [Ca\^2\textsuperscript{+}], (not shown).

To determine whether occupancy with uPA influences Ca\^2\textsuperscript{+} signaling through uPAR, uPA-occupied uPAR were selectively cross-linked with an anti-uPA primary mAb. As shown in Fig. 4, there was a relatively small increase from baseline to peak [Ca\^2\textsuperscript{+}], corresponding to a \Delta [Ca\^2\textsuperscript{+}], of 147 \pm 59.6 nM. Cross-linking a control IgG1 mAb had no effect (\Delta [Ca\^2\textsuperscript{+}], = 17.7 \pm 9.8 nM). When all available uPAR were fully saturated with uPA, cross-linking uPA then induced Ca\^2\textsuperscript{+} transients that were generally comparable to those induced by uPAR cross-linking, with larger \Delta [Ca\^2\textsuperscript{+}], that were statistically indistinguishable from the responses to cross-linking the anti-uPA mAb. The \Delta [Ca\^2\textsuperscript{+}], without uPA loading was 33% of the maximal \Delta [Ca\^2\textsuperscript{+}], (elicited by cross-linking uPA-loaded uPAR), which is essentially equivalent to the 30% occupancy rate of uPAR, as determined by flow cytometry. Therefore, magnitude of the [Ca\^2\textsuperscript{+}], signal appears to be related to the quantity of uPAR cross-linked regardless of the state of occupancy with uPA.

**Expression of adhesion molecules**

Many routes of proinflammatory signaling induce PMNs to alter the expression of adhesion molecules so as to facilitate the transition from nonadherent or loosely adherent, rolling adhesion to firm, integrin-mediated adhesion. To determine whether signals initiated by uPAR can promptly alter expression of adhesion molecules, PMNs were subjected to uPAR cross-linking, after which the cells were immunolabeled with FITC-conjugated anti-CD11b, FITC-conjugated anti-L-selectin, or an isotype-matched (IgG1) control mAb. Cross-linking uPAR increased surface levels of CD11b by \sim 80\%, with the maximal response occurring within 5 min (Fig. 5). While cross-linking the control IgG2a mAb induced a smaller increase in CD11b expression, the response to uPAR cross-linking was significantly greater after both 5 and 10 min (\textit{p} < 0.02). Progressive loss of L-selectin was seen over 40 min even after cross-linking the control IgG2a mAb, possibly triggered by the repeated manipulations inherent in the cross-linking procedure. Cross-linking uPAR caused greater losses of L-selectin consistently throughout the time course, but the differences from control were not statistically significant.

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**FIGURE 3.** A, Top, Representative tracing of [Ca\^2\textsuperscript{+}], demonstrating that exposing PMN to 4000 IU/ml of HMW-uPA triggers a prompt, but transient, change in [Ca\^2\textsuperscript{+}], (Fig. 2). Bottom, Pooled results for baseline, peak, and \Delta [Ca\^2\textsuperscript{+}], are shown (n = 8). B, The integrated Ca\^2\textsuperscript{+} signal (area under the curve) was \sim 3-fold greater in response to uPAR cross-linking (uPAR XL) than HMW-uPA.
Effects of uPA-uPAR aggregation on neutrophil degranulation

To further examine the downstream consequences of uPAR aggregation, PMNs were subjected to the receptor aggregation protocols described above and incubated at 37°C for 10 min in the presence of cytochalasin B (5 μg/ml) to enhance the release of intracellular granules (33). Cells were preloaded with fluo-3/AM so the increase in $[\text{Ca}^{2+}]_i$ could be monitored in parallel. As shown in Fig. 6, uPAR aggregation significantly increased the release of β-glucuronidase (a marker of azurophilic granules) and lactoferrin (a marker of specific granules). In both cases, degranulation was significantly greater than the response to cross-linking a control IgG2a mAb. Interestingly, the magnitude of both degranulation responses correlated very closely with the log of the concomitant $[\text{Ca}^{2+}]_i$ (Fig. 6). Selectively aggregating uPA-uPAR complexes with the anti-uPA mAb produces minimal levels of degranulation, again in keeping with the smaller increases in $[\text{Ca}^{2+}]_i$ (Fig. 7). However, preloading uPAR with exogenous HMW-uPA at 4°C before aggregating the uPA-uPAR complexes augmented the degranulation responses, virtually duplicating the effect of aggregating uPAR directly. Preloading with HMW-uPA followed by sham cross-linking (no primary mAb) did not induce degranulation (not shown). The effects of acutely exposing cells to high concentrations of HMW-uPA was determined by adding 4000 IU/ml to PMNs at 37°C and measuring degranulation over 10 min. HMW-uPA triggered a modest degree of degranulation over unstimulated controls ($3.7 \pm 2.1\%$ β-glucuronidase release (mean ± SEM; $n = 5$; not significant) and $214.3 \pm 67.9$ ng/ml lactoferrin release (mean ± SEM; $n = 6$; $p < 0.03$)). These findings indicate that both the magnitude of the $[\text{Ca}^{2+}]_i$ signal and the degranulation responses are dependent upon the extent to which uPAR is aggregated, and the occupancy state of uPAR with uPA has little or no impact on degranulation.

Modulation of PMN superoxide release

Freshly purified PMNs were treated to cross-link uPAR directly (3B10 anti-uPAR mAb, binding preferentially to unoccupied
or only uPA-uPAR complexes (using the anti-uPA mAb). Cells were then incubated 45 min to measure superoxide release. Experiments were also performed where cross-linking was initiated 30 min before adding FMLP, after which superoxide release was measured over 45 min. Neither saturating available uPAR with HMW-uPA (1 μg/ml) nor uPAR aggregation with the 3B10 mAb had any direct effect on superoxide production (Fig. 8). By contrast, selectively cross-linking uPA-uPAR complexes significantly increased superoxide release, approximately equivalent to a half-maximal response to FMLP. Moreover, the amount of uPA bound to uPAR, although occupying only 30% of the available binding sites, was sufficient to support this response fully, because superoxide release was not increased further by preloading uPAR with HMW-uPA before cross-linking. Aggregating uPA-uPAR complexes also increased the subsequent superoxide response to FMLP over a range of concentrations from 10⁻²–10⁻⁶ M (Fig. 9). At the higher FMLP concentrations, the additive effect was approximately twice the increment in superoxide release induced by uPA/uPAR cross-linking alone. Again, saturating uPAR with uPA before cross-linking with the anti-uPA mAb did not magnify this effect. Directly cross-linking uPAR with the 3B10 mAb enhanced FMLP-induced superoxide release as well, but the effect was comparatively small (Fig. 9). These findings indicate that aggregating uPA-uPAR complexes is capable of enhancing superoxide release both alone and as a costimulus with FMLP, while aggregating the receptor without its ligand cannot fully duplicate these responses.

**FIGURE 6.** Effects of cross-linking uPAR (uPAR XL) or an isotype-matched control mAb (IgG2a XL) on release of β-glucuronidase (A; percentage of total cell content > release by unstimulated cells) and lactoferrin (B; nanograms per milliliter > release by unstimulated control cells). The uPAR XL induced significantly greater release of both β-glucuronidase and lactoferrin than the IgG2a XL (mean ± SEM). In both instances, the magnitude of the degranulation response correlated significantly with the concomitant Δ [Ca²⁺]ₙ (log transformed).

**Discussion**

The mechanisms by which uPAR triggers activation signaling are only partly elucidated. In some cell types, uPAR is a component of large multiprotein complexes containing signaling intermediates such as Src and Jak tyrosine kinases (16–21). Structural characterization of uPAR has shown that it is a glycosylated single polypeptide linked to the plasma membrane by a GPI anchor (37). Several GPI-anchored proteins are known to regulate cellular activation, adhesion, and migration (38), even though GPI anchors only penetrate the outer leaflet of the plasma membrane and thus have no direct access to cytoplasmic signaling intermediates. The ability of uPAR to complex with membrane-spanning proteins has suggested that uPAR can initiate leukocyte activation by appropriating the signaling mechanisms of a partner protein such as CR3, in effect using it as a signal transduction device. It has been shown that cooperative signaling through uPAR and CR3 is required for exogenous uPA to generate an intracellular signal in human PMNs (22). By contrast, the Ca²⁺ signal induced by uPAR aggregation appears to be CR3 independent, although it remains to be determined what membrane-spanning partner proteins, if any, are necessary for this signaling pathway (27). Membrane-spanning proteins associated with uPAR include the β₅ integrins LFA-1, CR3, and CR4; β₇, β₈, and β₉ integrins (9–15, 39); and the mannose-6-phosphate/insulin-like growth factor II receptor (40). Exogenous uPA or its uPAR-binding fragment can trigger phosphorylation of multiple
intracellular proteins (including Src kinases), increase [Ca$^{2+}$], and activate fos, jun, and myc oncogenes (18–22, 27, 41–43). Engaging uPAR by uPA binding or by cross-linking also activates Jak1 tyrosine kinase and nuclear translocation of Stat1 transcription regulatory factor (16, 17). In some cell types, uPAR is found in or near caveolae, specialized structures of the plasma membrane where signaling molecules are congregated (44–46). In uPAR-transfected epithelial cells, caveolin, a major constituent of caveolae, serves as an organizing protein for Src kinase activation (46). It remains to be determined whether uPAR interacts with caveolin or functions within caveolae-like structures in leukocytes. The recently described coupling between uPAR and the mannose-6-phosphate/insulin-like growth factor II receptor may indirectly influence activation signaling under some circumstances by facilitating activation of latent TGF-$eta_1$, but it is not known whether a direct pathway of cooperative signaling between these proteins exists (40).

The present study demonstrates that uPAR, although expressed at relatively low levels in resting human PMNs, is capable, when aggregated, of initiating intracellular signaling, manifested by increased [Ca$^{2+}$]. The response to uPAR aggregation was substantial, one-half of the response elicited by FMLP (Figs. 1 and 2), as reflected by the integrated Ca$^{2+}$ signal (area under the Ca$^{2+}$ vs time curve), which was used to contend with the distinctly different Ca$^{2+}$ waveforms generated by the two stimuli. Analyzing the results in this way, it is also apparent that uPAR interacts with caveolae or functions within caveolae-like structures in leukocytes. The recently described coupling between uPAR and the mannose-6-phosphate/insulin-like growth factor II receptor may indirectly influence activation signaling under some circumstances by facilitating activation of latent TGF-$eta_1$, but it is not known whether a direct pathway of cooperative signaling between these proteins exists (40).

The immediate effects of aggregating uPAR include significant up-regulation of CR3 expression (Fig. 5), suggesting that uPAR aggregation may be a factor in the progression of PMNs toward firm, CR3-dependent adhesion. This transition is not uniquely elicited by uPAR cross-linking, as aggregating L-selectin and CR3 produce similar results (3–5). Nevertheless, CR3 and L-selectin colocalize during L-selectin aggregation, just as uPAR can colocalize with CR3, so it possible that these activation schemes are all converging on shared signaling pathways rather than representing redundant, but independent, mechanisms of PMN activation (3, 14).

As shown in Fig. 6, uPAR aggregation elicits PMN degranulation, as represented by enhanced release of $\beta$-glucuronidase and lactoferrin (48). The magnitude of the degranulation response was closely related to the magnitude of the increase in [Ca$^{2+}$], and neither was significantly affected by the state of receptor occupancy, i.e., the presence or the absence of associated uPA. Although few if any agonists can match the massive degranulation induced by FMLP, the magnitude of the degranulation response to uPAR aggregation was comparable to that described for PMN extravasating into a wound in vivo or to the responses in vitro to other proinflammatory agonists, such as leukotriene B4 and IL-8 (49–51). Because uPAR aggregation occurs as leukocytes adhere and migrate (7, 14), it is reasonable to speculate that coordinating with granule release could enhance vascular permeability and matrix degradation, and thereby facilitate PMN extravasation. The role of intracellular Ca$^{2+}$ mobilization in triggering PMN degranulation remains controversial, but it appears that increased [Ca$^{2+}$] is variably associated with degranulation depending on the agonist (52). The close correlation between the two events in this study certainly implies that in the case of uPAR aggregation, they are proximate downstream events of a common pathway.

**FIGURE 7.** Selective cross-linking of endogenously occupied uPAR with anti-uPA mAb has no effect on release of $\beta$-glucuronidase or lactoferrin (left), but saturating uPAR with HMW-uPA produces a significant increase in both responses to subsequent uPA cross-linking (right). Cross-linking an isotype-matched control IgG1 had no effect on degranulation regardless of prior HMW-uPA loading.
One of the more remarkable findings of this study was the enhanced superoxide release triggered specifically by aggregating uPA-uPAR complexes (Figs. 8 and 9). In many ways, this observation expands on the prior observations of Cao et al. (22), who reported that HMW-uPA primes PMNs for superoxide release by forming a ternary signaling complex with uPAR and CR3. The present study shows that aggregating uPA circumvents the need for very high, and arguably supraphysiologic, concentrations of HMW-uPA needed to elicit this response. In fact, the relatively small proportion (~30%) of uPAR occupied with uPA was fully elicited a significant increase in superoxide release. By contrast, preferentially cross-linking unoccupied uPAR with the 3B10 mAb (uPAR XL) and saturating with HMW-uPA had no effect (significance determined by one-way ANOVA with Bonferroni’s post-test; mean ± SEM; n = 5; unstimulated controls, n = 18).

Effects of uPAR cross-linking on superoxide release. Selectively cross-linking uPAR endogenously occupied with uPA (uPA-XL) or uPAR saturated with exogenous HMW-uPA (uPA + uPA XL) both elicited a significant increase in superoxide release. By contrast, preferentially cross-linking unoccupied uPAR with the 3B10 mAb (uPAR XL) and saturating with HMW-uPA had no effect (significance determined by one-way ANOVA with Dunnett’s post-test; mean ± SEM; n = 4; asterisk, p < 0.01).

Prior work has emphasized the role that uPAR plays as an adapter protein that articulates its effects on cellular functions through its interactions with integrins. The present study has examined uPAR clustering as an isolated stimulus for proinflammatory signaling in nonadherent PMNs. However, one would expect that activation signaling initiated by uPAR aggregation in vivo would occur in adherent PMNs. It is not yet clear how uPAR clustering occurs in vivo, but it may occur actively as PMNs encounter a uPAR counterligand such as vitronectin (54), or it may occur passively as integrins or other proteins capable of binding uPAR are drawn into a clustered configuration. In either case, the signaling pathways engaged by uPAR clustering may be coupled to complimentary chemokine and integrin-mediated signaling events, and it will be necessary to investigate the role of uPAR-mediated activation signaling in this context.

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References


