Soluble E-selectin Acts in Synergy with Platelet-activating Factor to Activate Neutrophil β2-Integrins

ROLE OF TYROSINE KINASES AND Ca2+ MOBILIZATION*

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Selectins play a critical role in neutrophil recruitment to sites of inflammation, in tethering and rolling of neutrophils on vascular endothelium, as well as triggering β2-integrin-mediated adhesion. We have previously demonstrated potential pro-inflammatory effects of soluble E-selectin upon neutrophil effector functions, using a soluble recombinant molecule (E-zz), which increased β2-integrin-mediated adhesion, decreased β2-integrin-dependent migration, and triggered reactive oxygen species generation and release. In this study, we have examined the intracellular signals following neutrophil activation by soluble E-selectin. We show that exposure of neutrophils to E-selectin and platelet-activating factor (PAF) in combination induced a synergistic effect upon β2-integrin-mediated adhesion. Although soluble E-selectin did not induce Ca2+ mobilization in neutrophils by itself, elevation of intracellular Ca2+ was specifically prolonged in response to PAF but not leukotriene B4 or N-formyl-Met-Leu-Phe. The prolonged Ca2+ mobilization observed in the presence of E-selectin was dependent on Ca2+ influx from intracellular stores rather than influx of extracellular Ca2+ through SKF 96365-sensitive channels. The specific alteration of Ca2+ mobilization reported here appears not to have a role in the synergistic effects of E-selectin and PAF upon neutrophil O2+ release but may be involved in augmentation of β2-integrin-mediated adhesion.

Recruitment of inflammatory cells in response to injury or infection involves coordinated regulation of specific receptor/counter-receptor pairs from the integrin, immunoglobulin, selectin, and mucin families (1). The control of events involved in leukocyte recruitment is critical for the development of effective anti-microbial defenses and also for efficient wound healing. Failure to recruit granulocytes is life threatening, resulting in inadequate clearance of opportunistic pathogens. However, excessive inflammatory cell recruitment or inappropriate cell activation leads to the development of chronic inflammatory conditions that may favor fibrotic repair mechanisms and, ultimately, loss of organ function (2).

It is now well established that a multistep molecular process allows leukocyte recruitment to different tissue sites. In acute inflammatory responses, neutrophil granulocyte recruitment involves L-selectin and P-selectin glycoprotein ligand-1 that engage endothelial counter-receptors including endothelial P- and E-selectin (3). Conversion of “rolling” adhesion to “firm” adhesion necessary for subsequent transmigration requires an additional co-stimulus, for example via intracellular signals from occupancy of G protein-coupled seven-transmembrane receptors for bacterial peptides, inflammatory mediators, and chemokines (4, 5). Trans-endothelial migration is clearly a complex process but is thought to involve dynamic modulation of integrin-mediated adhesion events to allow formation of new sites of contact at the leading edge of the cell and selective detachment at the trailing end (6–8). The existence of multiple levels of control for adhesion processes ensures that “inappropriate” recruitment of leukocytes does not occur. However, a breakdown in the normal adhesion regulatory mechanisms may contribute to pathogenesis of a number of diseases in which the granulocyte has been implicated (9).

Cellular recruitment is critically dependent on formation of molecular interactions between receptor/counter-receptor pairs. However, it is now clear that many adhesion receptors are also coupled to the intracellular signal transduction machinery, providing “outside-in” signals that modulate leukocyte responses. Initiation of signal transduction pathways following integrin occupancy is relatively well established (10). For example, release of degradative enzymes and toxic oxidant species required for antimicrobial defense is augmented following adhesion to matrix components. However, the role of other adhesion receptors in triggering signaling events has only recently begun to be defined. Engagement of CD31 has been demonstrated to augment the rate of integrin-dependent migration, providing a mechanism for ensuring efficient diapedesis (11). Recent evidence also suggests that engagement of receptors involved in rolling adhesion may also transduce specific signals that alter neutrophil behavior. In particular, ligation of neutrophil L-selectin and P-selectin glycoprotein ligand-1 augment β2-integrin-mediated adhesion and increase production of damaging reactive oxygen intermediates (12, 13). Selectin-dependent alterations in neutrophil function have been suggested to require co-stimulation with other agents, e.g. platelet-activating factor (PAF)1 (14, 15). The functional alter-

* This work was supported by the Scottish Hospital Endowment Research Trust and by Arthritis Research Campaign Grant D0570. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: PAF, platelet-activating factor; TNF-α, tumor necrosis factor-α; M LP, formyl-Met-Leu-Phe; LT B4, leukotriene B4; PMA, phorbol 12-myristate 13-acetate; ACLB, albumin-coated latex bead(s); HBSS, Hank’s balanced salt solution; AM, acetoxymethyl ester; IP3, inositol trisphosphate.
ations induced by agents that activate neutrophils appear distinct from those caused by "priming" agents, which have little effect on generation of reactive oxygen species by themselves. Together these findings indicate that the repertoire of receptors engaged on the neutrophil surface determines the functional responses in terms of adhesion, migration, and secretion. One implication is that although it may be possible to define experimental conditions in vitro under which neutrophils are "unprimed," "primed," or "activated," the functional status of neutrophils during recruitment in vivo may approximate to a continuum of activation states.

Our previous studies have demonstrated that neutrophils treated with soluble recombinant E-selectin (E-zz) comprising the N-terminal C-type lectin domain, epidermal growth factor domain and two of the short consensus repeats with two protein A domains in tandem cause increased β2-integrin-mediated adhesion with a concomitant decrease in β2-integrin-dependent migration. In addition, generation and release of reactive oxygen species is increased following exposure to E-selectin (16). These effects could be blocked by F(ab')2 anti-E-selectin monoclonal antibody binding to the lectin domain of E-selectin. The kinetics of the neutrophil response to E-zz raised the possibility that the effects might be indirect because of secondary release of inflammatory mediators from neutrophils, for example PAF. In this paper we examine the generation of intracellular signals following exposure of neutrophils to E-selectin. We have found that E-selectin specifically prolongs [Ca2+]i increase in neutrophils treated with PAF but not N-formyl-Met-Leu-Phe (fMLP) or leukotriene B4 (LTB4). In addition, we have addressed whether soluble E-selectin effects were due to a second release of PAF and found that soluble E-selectin was not acting directly through PAF receptors. However, our data suggest that specific changes in [Ca2+]i are not required for E-selectin-dependent augmentation of functional responses in neutrophils.

EXPERIMENTAL PROCEDURES

Soluble Recombinant Proteins and Other Reagents—Recombinant human E-selectin (E-zz) and recombinant human vascular adhesion molecules (V-zz) were provided by Michael Bird and Carmel Horgan (Glaxo Wellcome, UK) as described previously (16). Hanks' balanced salt solution (HBSS) was obtained from Life Technologies, Inc. Dextran T500 was obtained from Amersham Pharmacia Biotech. PAF, fMLP, PMA, TNF-α, interleukin-8, cytochrome c, superoxide dismutase, genistein, and digitonin were obtained from Sigma. Ro-318220, SKF 96365, and TMB-8 were from Calbiochem-Novabiochem Ltd (Nottingham, UK). UK-74505 was a gift from Dr. M. J. Parry (Pfizer Central Research, Sandwich, Kent). Fura-2-AM was obtained from Molecular Probes (Eugene, OR; supplied by Cambridge Bioscience, Cambridge, UK).

Neutrophil Isolation—Polymorphonuclear leukocytes were isolated from peripheral blood of healthy donors as described previously (18). Briefly, after centrifugation of citrated whole blood at 3000 × g for 20 min and removal of platelet-rich plasma, leukocytes were separated from erythrocytes by dextran sedimentation using 0.6% dextran T500. Polymorphonuclear leukocytes were then separated from mononuclear leukocytes using discontinuous isotonic Percoll gradients. Polymophonuclear leukocytes were 95–98% neutrophils using morphological criteria, and viability was >99% as assessed by trypan blue exclusion.

Neutrophil Adhesion to Albumin-coated Latex Beads—β2-Integrin-dependent adhesion of ACLB to freshly isolated neutrophils was measured as described previously (17). Fluorescent latex beads were washed in HBSS and incubated with 10 mg/ml human serum albumin for 15 min before washes in HBSS and resuspension at 0.5% in HBSS. Neutrophils (155 µl at 107/ml in HBSS in the presence or absence of E-zz (5 µM)) were added to 25 µl of ACLB and 25 µl of albumin as described under "Results." For inhibition experiments, neutrophils were preincubated with TMB-8 (250 µM), SKF 96365 (5 µM), or UK-74505 (1 µM) for 10 min before addition of ACLB. Cells and ACLB were then incubated at 37 °C for 15 min in a shaking water bath at 110 beats/min. After fixation of the cells with 0.5 ml of 0.5% glutaraldehyde, nonadherent ALCB were removed by three washes. Bead binding to neutrophils was measured by flow cytometry using a FacsCalibur (Becton Dickinson, Oxford, UK).

Determination of [Ca2+]i, by Spectrofluorimetry—Freshly isolated neutrophils were incubated at 107/ml in Ca2+- and Mg2+-free HBSS with 2 µM Fura-2-AM for 15 min at 37 °C. Cells were suspended at 2 × 106/ml in HBSS containing Ca2+ and Mg2+ essentially as described (18). A SPEX Fluoromax spectrofluorimeter with excitation wavelengths at 340 and 380 nm and an emission wave-length recorded at 510 nm was used for measuring [Ca2+]i. [Ca2+]i was calculated as described (18, 19) using the equation: [Ca2+]i = Kd × B × (r - rmin)/rmax - r, where r is the measured Fura-2 fluorescence ratio between 340 and 380 nm. rmax is the maximum 340/380 nm ratio, was obtained by lysing cells with 25 µM digitonin. rmin, the minimum 340/380 ratio, was obtained by addition of 10 mM EGTA. Kd is the dissociation constant of the Fura-2/Ca2+ complex (224 nm), and B is the ratio of fluorescence at 380 nm at 0 and saturating Ca2+ concentration.

Measurement of Superoxide Anion Release—The determination of release of superoxide anions by freshly isolated neutrophils was performed as described previously (16). Briefly, neutrophils were preincubated with genistein (74 µM) or Ro-318220 (1 µM) for 15 min at 37 °C before stimulation with soluble recombinant proteins, FMLP, or PMA in the presence of cytochrome c (1 mg/ml). After 15 min at 37 °C, the reaction was stopped by placing the cells on ice, followed by a centrifugation (13,000 × g, 2 min, 4 °C). The superoxide dismutase (200 units) sensitive reduction of cytochrome c was determined for each supernatant by measuring the peak absorbance between 555 and 565 nm using a Pye-Unicam scanning spectrophotometer. Results are expressed as nanomoles of superoxide anions generated per 10⁶ neutrophils.

Statistical Analysis—Using the paired Student’s t test, the difference between the mean of the assays and control groups was considered significant when p < 0.05. The results are expressed as the means ± S.D. of the number (n) of independent experiments each using cells isolated from separate donors.

RESULTS

Requirement for Ca2+ in E-selectin-mediated Augmentation of β2-Integrin Adhesion—Our previous studies demonstrated that there was a temporal association between the extent of augmentation of neutrophil effector function and the duration of treatment with soluble E-selectin (E-zz), raising the possibility that E-zz triggered secondary release of inflammatory mediators, e.g. PAF. We therefore tested whether the PAF antagonist UK-74505 affected E-zz-induced β2-integrin-mediated adhesion using a well characterized assay involving binding of albumin-coated latex beads (16, 17). Although UK-74505 blocked PAF-induced β2-integrin activation, it had no effect on β2-integrin activation following treatment with either FMLP or E-zz (Fig. 1A), suggesting that E-zz effects are independent of PAF release and ligation of the PAF receptor.

Careful analysis of the effects of E-zz and PAF alone or in combination demonstrated that treatment of neutrophils with both agents induced a synergistic effect upon β2-integrin activation (Fig. 1B). Thus, although PAF (0.1 nM) or E-zz alone resulted in a small augmentation of β2-integrin-mediated adhesion (1.8- and 3.5-fold, respectively), the combination resulted in 8.6-fold increase in neutrophil β2-integrin activation to a level comparable with that seen with 10 nM FMLP. Because changes in [Ca2+]i, are an important early event following ligation of the seven-transmembrane receptor family (20, 21), we next assessed whether E-zz affected the PAF response by altering Ca2+ fluxes within neutrophils by using different Ca2+ channel inhibitors. Treatment of neutrophils with SKF 96365 (5 µM), a receptor-operated channel inhibitor, did not block augmentation of β2-integrin activity with E-zz alone or in combination with PAF and only partially blocked β2-integrin-mediated adhesion induced by PAF at 10 nM. In contrast, treatment of neutrophils with 250 µM TMB-8, an intracellular calcium antagonist, completely abolished E-zz- and PAF-mediated β2-integrin activation (Fig. 1B). FMLP-induced β2-integrin activation was not significantly inhibited by TMB-8 (Fig. 1B), indicating that Ca2+ mobilization is not required for induction.
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Fig. 1. Requirement for Ca\textsuperscript{2+} in E-selectin-mediated augmentation of \(\beta_2\)-integrin adhesion. Freshly isolated neutrophils were preincubated with UK-74505 (1 \(\mu\)M) (A) or TMB-8 (250 \(\mu\)M) or SKF 69365 (5 \(\mu\)M) or HBSS only for 10 min at 37 °C before addition of ACLB, in the presence of E-zz (5 \(\mu\)g/ml), PAF (0.1 nM or 10 nM), fMLP (10 nM), or both E-zz and PAF (0.1 nM) (B). After 15 min at 37 °C, neutrophils were fixed with glutaraldehyde, and nonadherent ACLB were removed by three washes. Neutrophil binding to ACLB was measured by flow cytometry using a FacsCalibur (Becton Dickinson). Data are the means from three separate experiments.

Soluble E-selectin Prolongs PAF-induced Calcium Mobilization—The distinct specific effects of TMB-8 upon \(\beta_2\)-integrin activation induced by PAF and E-zz led us to consider whether E-zz might act to alter Ca\textsuperscript{2+} mobilization within neutrophils in response to PAF. In contrast to the rapid and transient elevation of [Ca\textsuperscript{2+}], in response to PAF, treatment of neutrophils with E-zz alone did not induce any changes in [Ca\textsuperscript{2+}], (Fig. 2A), indicating that E-zz may act through a pathway distinct from IP\textsubscript{3}-dependent Ca\textsuperscript{2+} mobilization. Interestingly, preincubation of neutrophils with E-zz did not affect the initial increase of [Ca\textsuperscript{2+}] in response to PAF but caused a subsequent sustained increase in [Ca\textsuperscript{2+}] (Fig. 2A). Preincubation of neutrophils with V-zz did not affect [Ca\textsuperscript{2+}], in response to PAF (Fig. 2A), indicating that the effects of E-zz upon calcium mobilization are specific. Analysis of the concentration responses revealed that E-zz was maximally effective following treatment with higher concentrations of PAF (Fig. 2B), suggesting that a threshold increase in [Ca\textsuperscript{2+}] is required above which E-zz prolongs calcium mobilization.

PAF-induced Ca\textsuperscript{2+} mobilization was ablated by TMB-8 (Fig. 3A), indicating that release of Ca\textsuperscript{2+} from intracellular stores may be an essential trigger for PAF-induced binding to ACLB. The prolongation of [Ca\textsuperscript{2+}] elevation in the presence of E-zz was independent of SKF 96365-sensitive Ca\textsuperscript{2+} channels (Fig. 3, B and C). Thus, although SKF 96365 blocked PAF-induced extracellular Ca\textsuperscript{2+} influx, Ca\textsuperscript{2+} mobilization in the presence of E-zz was relatively insensitive to SKF 96365. Together with data presented in Fig. 1, these results indicate that E-zz treatment may affect PAF responses via the release of Ca\textsuperscript{2+} from intracellular stores rather than influx of extracellular Ca\textsuperscript{2+} through SKF 96365-sensitive receptor-operated channels.

Specificity of Soluble E-selectin Effects—We next considered whether treatment of neutrophils with E-zz prolonged Ca\textsuperscript{2+} mobilization in response to other agonists that act via distinct seven-transmembrane \(\alpha\) protein-coupled receptors. As shown in Fig. 4, in contrast with the effects of PAF, changes in [Ca\textsuperscript{2+}] following stimulation with either LTB\textsubscript{4} or fMLP were not affected by preincubation with E-zz. These findings raise the possibility that sustained elevation of [Ca\textsuperscript{2+}] observed when E-zz-treated cells were stimulated with PAF might account for the synergistic effects on \(\beta_2\)-integrin-mediated adhesion. We therefore tested whether \(\beta_2\)-integrin activation observed following LTB\textsubscript{4} or fMLP treatment was affected by preincubation with E-zz. As shown in Fig. 5, E-zz treatment alone induced a small but significant increase in \(\beta_2\)-integrin-mediated adhesion. The \(\beta_2\)-integrin activation response of neutrophils to LTB\textsubscript{4} and fMLP was not significantly affected by E-zz pretreatment, whereas E-zz and PAF together induced a significant increase in the percentage of cells that were capable of \(\beta_2\)-
Although integrin activation following E-zz/PAF treatment may parallel prolonged Ca\textsuperscript{2+} influx, analysis of the effects of E-zz upon neutrophil O\textsubscript{2}\textsuperscript{−} release provides evidence that E-zz effects were independent of Ca\textsuperscript{2+} mobilization. Classical priming agents such as PAF or TNF-\alpha have no effect upon neutrophil superoxide release when used alone but prime release in response to a second stimulus such as fMLP or the chemokine interleukin-8. Untreated neutrophils release little O\textsubscript{2}\textsuperscript{−} (1.08 ± 0.36 nmol O\textsubscript{2}\textsuperscript{−}/10\textsuperscript{6} cells), and fMLP stimulation alone does not increase the level of O\textsubscript{2}\textsuperscript{−} production (1.2 ± 0.35 O\textsubscript{2}\textsuperscript{−}/10\textsuperscript{6} cells), confirming that the neutrophils used in these experiments were minimally activated or unprimed (Fig. 6). Similarly, treatment with TNF-\alpha alone did not induce neutrophil O\textsubscript{2}\textsuperscript{−} release, but preincubation with TNF-\alpha caused a 7-fold increase in fMLP (100 nM)-induced O\textsubscript{2}\textsuperscript{−} release (Fig. 6). In contrast, E-zz treatment alone significantly induces O\textsubscript{2}\textsuperscript{−} release, and preincubation of neutrophils with E-zz prior to fMLP treatment resulted in a further two-fold increase in O\textsubscript{2}\textsuperscript{−} release to levels seen with TNF-\alpha and fMLP in combination (Fig. 6). Similar effects were seen when interleukin-8 (10 ng/ml) or PAF (0.1 nM) was used in combination with E-zz (data not shown), demonstrating that distinct mechanisms underlie the effects of integrin-mediated adhesion.

Fig. 3. The prolonged [Ca\textsuperscript{2+}], elevation induced by soluble E-selectin depends on Ca\textsuperscript{2+} influx from intracellular stores and is independent of SKF 96365-sensitive Ca\textsuperscript{2+} channels. Freshly isolated neutrophils were loaded with Fura-2-AM (2 \muM) and preincubated with (\textbullet) or without (\textsquare) E-zz (5 \mug/ml) for 15 min before measuring [Ca\textsuperscript{2+}]i. 24 s after recording, the cells were stimulated with PAF, LTB\textsubscript{4}, or fMLP, all used at 10 nM. Data are representative of five separate experiments.

Fig. 4. Soluble E-selectin effect upon Ca\textsuperscript{2+} mobilization is specific to PAF. Freshly isolated neutrophils were loaded with Fura-2-AM (2 \muM) and preincubated with or without E-zz (5 \mug/ml) for 15 min before measuring [Ca\textsuperscript{2+}]i. 24 s after recording, the cells were stimulated with PAF, LTB\textsubscript{4}, or fMLP, all used at 10 nM. Data are representative of three separate experiments.
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FIG. 5. β2-Integrin activation is affected by soluble E-selectin following PAF stimulation but not LTβR or fMLP. Freshly isolated neutrophils with ACLB were stimulated with PAF, LTβR, or fMLP at different concentrations as indicated, in the presence (●) or absence (■) of E-zz (5 μg/ml). Neutrophil β2-integrin-mediated binding of ACLB was determined by flow cytometry as for Fig. 1. Data are the means of three experiments.

FIG. 6. Soluble E-selectin effects upon O2− release involve distinct mechanisms when compared with priming agents such as TNF-α. Freshly isolated neutrophils were preincubated with E-zz or V-zz (5 μg/ml) for 15 min at 37 °C and then incubated with E-zz (5 μg/ml) or PMA (10 nM) for another 15 min at 37 °C in presence of cytochrome c (1 mg/ml). Superoxide dismutase-inhibitable reduction of cytochrome c was then determined by measuring the peak absorbance between 535 and 565 nm using a Pye-Unicam scanning spectrophotometer. Note the different scale for PMA response (at right on the graph). Data are expressed as nmol of O2/10⁶ cells and are shown as the means of three separate experiments.

E-zz upon neutrophil O2− release when compared with classical priming agents such as TNF-α. These data also suggest that the effects of E-zz upon neutrophil superoxide production and release are largely independent of effects upon Ca2+ mobilization. First, although E-zz alone does not elevate [Ca2+]i, significant amounts of neutrophil O2− are released in response to E-zz (compare Figs. 2A and 6). Second, our data indicate that E-zz does not affect Ca2+ mobilization in response to fMLP (Fig. 4), yet E-zz is able to convert fMLP from a nonsecretory to a secretory stimulus in terms of O2− release (Fig. 6). Together with our data relating to the effects of E-zz on β2-integrin-mediated adhesion, we conclude that the specific alteration of Ca2+ mobilization reported here does not have a role in the synergistic effects of E-zz and PAF upon neutrophil O2− release but is involved in β2-integrin-mediated adhesion.

Superoxide Anion Release Induced by Soluble E-selectin Is Tyrosine Phosphorylation-dependent—To further define the signaling pathways involved in neutrophil activation following soluble E-selectin treatment, we next tested the effect of inhibitors of protein kinases involved in intracellular signaling upon E-zz-induced neutrophil O2− release. E-zz-induced neutrophil O2− release was inhibited by genistein (74 μM), a potent tyrosine kinase inhibitor, but not by Ro-318220 (1 μM), a protein kinase C inhibitor (Fig. 7). In contrast, PKC-dependent O2− release induced by the phorbol ester (PMA) was blocked by Ro-318220 and not by genistein. These results demonstrate that Ro-318220 is indeed active in our assay, because PMA-stimulated O2− release is mediated predominantly via PKC activation. In addition, our results show that genistein is not causing a global nonspecific inhibition of neutrophil responsiveness. These findings suggest that activation of the NADPH oxidase following E-zz treatment is PKC-independent but requires protein tyrosine kinase activity. We are currently investigating specific targets of protein tyrosine kinases in neutrophils, which may be associated with altered neutrophil effector function in response to E-zz treatment.

DISCUSSION

Appropriate recruitment of neutrophils to sites of infection or tissue injury is critical for the initiation and progression of the inflammatory response. A number of studies have shown the importance of selectins in the orchestration of adhesion and migration of neutrophils in the initial capture and subsequent rolling on vascular endothelial ligands (22–24). Selectins also have a more complex regulatory role in inflammatory processes, initiating intracellular signal transduction cascades...
that lead to neutrophil activation and altered functional responses (12, 25, 26, 28–30). We have previously shown that soluble E-selectin (E-zz) specifically potentiates neutrophil adhesion and augments reactive oxygen species production and release (16). In this paper we further examine the nature of the signaling pathway that may underlie these regulatory effects.

The data presented here demonstrate several important findings relating to altered functional status following E-zz treatment, which are consistent with the concept of a juxta-crine activation process demonstrated for neutrophil-platelet interactions involving PAF and P-selectin (15). First, in support of our suggestion that elevated levels of soluble E-selectin may cause alterations in neutrophil behavior that are pro-inflammatory, E-zz is able to markedly alter neutrophil production of reactive oxygen species. Unlike classical priming agents (PAF or TNF-α), E-zz is able to trigger O2•− release when used alone. However, in combination with stimuli that do not normally trigger O2•− release from unprimed carefully prepared neutrophils (for example the bacterial peptide (MLP), E-zz potentiates O2•− production (Fig. 6). Second, we have observed that E-zz acts synergistically with PAF but not ILMP or LTB4 to augment β2-integrin activity, providing a mechanism for alteration of the adhesion repertoire of neutrophils exposed to specific inflammatory mediators at sites of extravasation. Third, analysis of the effects of E-zz upon Ca2+ mobilization within neutrophils revealed that E-zz specifically prolongs elevation of [Ca2+]i, in response to PAF but not ILMP or LTB4. It has been suggested that [Ca2+]i elevation was a necessary step in the signaling between the FMLP receptor and NADPH oxidase (20). Our data show that Ca2+ mobilization is not required for ILMP-induced β2-integrin activation. Moreover, we have shown that the effect of E-zz upon PAF-induced β2-integrin adhesion is dependent on a threshold increase in [Ca2+]i, and is blocked when neutrophils are treated with an intracellular Ca2+ antagonist (Fig. 1B). The observed dissociation between the effects of E-zz on β2-integrin activation, augmentation of O2•− release, and Ca2+ mobilization in response to PAF indicates that altered Ca2+ mobilization following PAF receptor ligation is not important for the synergistic action of PAF and E-zz upon O2•− release.

We have examined a number of parameters of neutrophil activation that might be affected by changes in PAF-induced Ca2+ mobilization, including the extent and duration of neutrophil polarization and whether transient β2-integrin mediated adhesion responses are prolonged following E-zz treatment. Although we have observed synergism between E-zz and PAF in terms of β2-integrin-mediated adhesion, the precise relationship between these events is difficult to determine. Because E-zz induces a low level of β2-integrin activation by itself yet fails to induce Ca2+ mobilization, a distinct functional correlate of prolonged Ca2+ elevation following E-zz and PAF treatment remains to be defined. One possibility is that [Ca2+]i elevation may have important consequences for arachidonic acid metabolism. Treatment of macrophages with PAF required a sustained increase in [Ca2+]i, to induce arachidonic acid release (32). Although the precise mechanisms of E-zz action upon PAF-induced Ca2+ mobilization remain to be determined, it would be interesting to investigate whether neutrophil arachidonic acid metabolism is affected by E-zz/PAF.

We are currently investigating the effects of E-zz upon neutrophil migration in response to PAF, where prolonged elevation of [Ca2+]i, may interfere with the dynamic activation/deactivation cycles of integrin ligand binding that are necessary for an efficient migration.

PAF induces an initial rapid increase in [Ca2+]i, because of IP3-dependent release of Ca2+ from intracellular stores localized in the endoplasmic reticulum (33), which is blocked by TMB-8 and is essential for E-zz effect upon PAF-induced Ca2+ mobilization. The second phase of PAF response is due to Ca2+ influx from extracellular sources through receptor-operated channels. The effects of E-zz upon PAF-induced Ca2+ mobilization was not blocked by SKF 96365, indicating that the sustained [Ca2+]i elevation following incubation with E-zz was not due to extracellular Ca2+ entry through SKF 96365-sensitive receptor-operated channels. Alternatively, because the E-zz effect requires a threshold increase in [Ca2+]i, following Ca2+ release from IP3-dependent intracellular stores, the sustained changes in [Ca2+]i, observed in the presence of E-zz could be due to extracellular Ca2+ entering the cells via store-operated channels (34), which are primed by E-zz and activated by intracellular Ca2+ release from endoplasmic reticulum. One hypothesis for the mechanism of activation of store-operated channels is the release of an intracellular messenger from the endoplasmic reticulum (for example arachidonic acid generated by the activation of phospholipase A2), which would bind to the store-operated channels and increase the probability of opening of the channels (35, 36). Another possibility would be an effect of E-zz upon intracellular Ca2+ stores, which may be insensitive to IP3.

Inhibition of E-zz effects upon neutrophil O2•− release by genistein suggests that tyrosine phosphorylation represents a critical step in E-selectin-mediated signaling, possibly controlling downstream functional events, such as the oxidative burst. Although L-selectin-induced Ca2+ mobilization has been reported to by tyrosine phosphorylation-dependent (28), we show for the first time that tyrosine phosphorylation is required for E-selectin induced signaling. Whether the sustained [Ca2+]i elevation following E-zz preincubation requires tyrosine phosphorylation has been difficult to assess, partly because PAF-induced Ca2+ mobilization itself is suppressed by tyrosine kinase inhibitors (data not shown). One possibility is that suppression of PAF-induced [Ca2+]i elevation was not sufficient to trigger E-zz-induced prolongation of [Ca2+]i elevation (Fig. 2B). Cross-linking of neutrophil L-selectin induced a rapid and transient [Ca2+]i increase and O2•− generation, which were both inhibited by genistein (37), indicating that activation of NADPH and Ca2+ mobilization may be downstream of the early signal cascade and require protein tyrosine kinase activation.

In conclusion, we present data that soluble E-selectin can specifically alter PAF induced Ca2+ mobilization in neutrophils but has little effect on Ca2+ mobilization in response to other agonists. Exposure of neutrophils to soluble E-selectin also alters the capacity for production of damaging reactive oxygen species in a manner that is distinct from classical priming agents. In addition, neutrophil β2-integrin-mediated adhesion is altered following exposure to E-selectin, with some degree of synergy observed when E-selectin-treated neutrophils are stimulated with other agonists, e.g. PAF. It is possible that engagement of E-selectin receptors on neutrophils may act physiologically to fine tune processes involved in adhesion and emigration. In view of recent findings that ligation of other neutrophil receptors such as CD31 (11), L-selectin (27), or ICAM-3 (31) influences either β2-integrin-mediated adhesion and/or production and release of reactive oxygen species, it seems likely that the repertoire of adhesion receptors engaged provides regulatory signals that determine neutrophil functional status.

Acknowledgments—We are very grateful to Dr. M. Bird and C. Horgan (Glaxo Wellcome, UK) for the generous gift of the recombinant human E-selectin and vascular cell adhesion molecules.
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