5-Oxo-ecosanoids and Hematopoietic Cytokines Cooperate in Stimulating Neutrophil Function and the Mitogen-activated Protein Kinase Pathway*

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The newly defined eicosatetraenoates (ETEs), 5-oxoETE and 5-oxo-15(OH)-ETE, share structural motifs, synthetic origins, and bioactions with leukotriene B4 (LTB4). All three eicosanoids stimulate Ca²⁺ transients and chemotaxis in human neutrophils (PMN). However, unlike LTB4, 5-oxoETE and 5-oxo-15(OH)-ETE alone cause little degranulation and no superoxide anion production. However, we show herein that, in PMN pretreated with granulocyte-macrophage or granulocyte colony-stimulating factor (GM-CSF or G-CSF), the oxoETEs become potent activators of the last responses. The oxoETEs also induce translocation of secretory vesicles from the cytosol to the plasmalemma, an effect not requiring cytokine priming. To study the mechanism of PMN activation in response to the eicosanoids, we examined the activation of mitogen-activated protein kinase (MAPK) and cytosolic phospholipase A2 (cPLA2). PMN expressed three proteins (40, 42, and 44 kDa) that reacted with anti-MAPK antibodies. The oxoETEs, LTB4, GM-CSF, and G-CSF all stimulated PMN to activate the MAPKs and cPLA2, as defined by shifts in these proteins’ electrophoretic mobility and tyrosine phosphorylation of the MAPK. However, the speed and duration of the MAPK response varied markedly depending on the stimulus. 5-OxoETE caused a very rapid and transient activation of MAPK. In contrast, the response to the cytokines was rather slow and persistent. PMN pretreated with GM-CSF demonstrated a dramatic increase in the extent of MAPK tyrosine phosphorylation and electrophoretic mobility shift in response to 5-oxoETE. Similarly, 5-oxoETE induced PMN to release some preincorporated [14C]arachidonic acid, while GM-CSF greatly enhanced the extent of this release. Thus, the synergism exhibited by these agents is prominent at the level of MAPK stimulation and phospholipid deacylation. Pertussis toxin, but not Ca²⁺ depletion, inhibited MAPK responses to 5-oxoETE and LTB4, indicating that responses to both agents are coupled through G proteins but not dependent upon Ca²⁺ transients. 15-OxoETE and 15(OH)-ETE were inactive while 5-oxo-15(OH)-ETE and 5(OH)-ETE had 3- and 10-fold less potency than 5-oxoETE, indicating a rather strict structural specificity for the 5-keto group. LY 255283, a LTB4 antagonist, blocked the responses to LTB4 but not to 5-oxoETE. Therefore, the oxoETEs do not appear to operate through the LTB4 receptor. In summary, the oxoETEs are potent activators of PMN that share some but not all activities with LTB4. The response to the oxoETEs is greatly enhanced by pretreatment with cytokines, indicating that combinations of these mediators may be very important in the pathogenesis of inflammation.

Stimulated cells release arachidonic acid and convert it to oxygenated products. Some of these, the thromboxanes, prosta glandins, and peptido-LTs, activate platelets, smooth muscle, and vascular endothelium. Others, the HETEs, lipoxins, hepxolins, and LTB4 target granulocytes (1–5). The many eicosanoids cooperate with each other, and with PAF and cytokines to coordinate function among the diverse cells mediating immunity (1–7). LTB4 acts on PMN at nanomolar levels while HETEs, lipoxins, and hepxolins are ≈100-fold less potent (1–4). Therefore, LTB4 may be the crucial eicosanoid for recruiting PMN. 5-HETE causes little or no PMN degranulation or O₂⁻ production but at submicromolar concentrations potentiates PAF in eliciting these responses. Even without PAF, it induces PMN to mobilize Ca²⁺, aggregate, and migrate (8–12). More importantly, 5-oxoETE and 5-oxo-15(OH)-ETE have these same actions but possess far greater potency than 5-HETE (11–13). These novel 5-HETE derivatives were found to be active at nanomolar concentrations. The two oxoETEs are also potent chemoattractive factors for eosinophils (14, 15).

5-HETE is often a dominant metabolite of arachidonic acid (15–17). For instance, PMN, while usually producing more LTB4 than 5-HETE, make predominantly 5-HETE if stimulated in the presence of cytokines, platelets, or erythrocyes (18–21). PMN as well as eosinophils and monocytes also make 5-oxoETE and 5-oxo-15(OH)-ETE. While regulation of the synthesis of these products is not fully understood, PMN, eosino-

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phils, and monocytes have a dehydrogenase activity that converts 5-hydroxy eicosanoids to their 5-oxo counterparts (15, 22-24). Other enzymatic pathways and free radical reactions also produce oxoETEs (11). Thus, multiple routes may result in 5-oxoETE formation. 5-HETE shares structural motifs with LTB₄ and indeed displaces it from its receptors (9). Nonetheless, most of the known effects of 5-HETE on PMN do not involve LTB₄ receptors (9–13). In reflection of this, 5-HETE has actions that LTB₄ either lacks or has to a lesser extent. 5-HETE increases the potency of PAF on PMN more than LTB₄ given 5-HETE's ability to compete with LTB₄ binding to its receptors (12, 13). Other enzymatic pathways and free radical reactions convert 5-hydroxy eicosanoids to their 5-oxo counterparts (15, 25–27), and intensifies smooth muscle contraction responses (28, 29). In addition, 5-HETE increases the amount of histamine released by mast cells (30) and stimulates many LTB₄-insensitive cells (11, 15). 5-OxoETE and 5-oxo-15(OH)-ETE desensitize PMN to 5-HETE but not to LTB₄ or other chemotactic factors (8–11). Therefore, the three 5-oxo/OH-ETEs apparently comprise a family of mediators that act on a unique recognition system. However, the function of this family of compounds is not known. For example, it was unknown whether 5-oxoOH-ETEs mobilize secretory vesicles or cooperate with hematopoietic cytokines. Such activities would facilitate chemotactic factor recruitment of PMN. Secretory vesicles contain integrins that attach PMN to vascular endothelium, while cytokines enhance PMN responses to chemotactic factors. Should 5-oxoOH-ETEs mimic cytokines in these respects, they too might trigger PMN entry into tissues and cooperate with inflammatory cytokines in vivo. For any such action, it is still incumbent to define the role of LTB₄ receptors, given 5-HETE's ability to compete with LTB₄ binding to its receptor on PMN.

We also know relatively little about how 5-oxoOH-ETEs stimulate cells. FMLP and PAF operate via receptors to activate heterotrimeric G proteins, raise cytosolic Ca²⁺, and activate MAPK (31–40). MAPK in turn activates cPLA₂ (41), which then releases arachidonic acid from phospholipids and likely initiates PAF synthesis (25). 5-OxoOH-ETEs require G proteins and stimulate Ca²⁺ transients (8–11) but differ from chemotactic factors in that the excitation they cause does not lead to appreciable arachidonic acid release or PAF synthesis (25). Thus, the influence of 5-oxoOH-ETEs on MAPK and cPLA₂ remained to be determined. In this report, we describe the effects of 5-oxoOH-ETEs on degranulation, O₂ production, secretory vesicles, MAPK activation, and cPLA₂ activation. In addition, we report the synergistic effects of pretreatment with cytokines before stimulation with 5-oxoETE.

**EXPERIMENTAL PROCEDURES**

**Reagents and Buffers**—We synthesized the oxo-eicosanoids by our published procedures (13). We purchased polyclonal rabbit antibody to rat ERK-1 carboxyl-terminal peptide (residues 333–367; anti-ERK-1), rabbit polyclonal antibody to rat ERK-1 subdomain 11 (residues 305–327; anti-ERK-1 K23), murine monoclonal antibody to rat ERK-2 (Upstate Biotechnology Inc., Lake Placid, NY; anti-ERK-2), rabbit antibody to the carboxyl-terminal peptide (residues 345–350) of rat ERK-2 (Santa Cruz Biotechnology, Santa Cruz, CA; anti-ERK-2 C14), and rabbit polyclonal antibody to residues 196–209 (DHTGFLTEYP)VATRWC of tyrosine phosphorylated human ERK-1 (New England Biolabs, Beverly, MA; anti-ERK-1 Y(PO)₃). The anti-ERK-1 Y(PO)₃ recognizes tyrosine-phosphorylated but not unphosphorylated ERK-1 or ERK-2. We also purchased horseradish peroxidase-conjugated polyclonal antibody to IgG (Transduction Labs, Lexington, KY); 0.45 m nitrocellulose membranes, and ECL kits (Amersham Corp.); PhosphoPlus MAPK antibody kits (New England Biolabs); phenylmethylsulfonyl fluoride, diisopropyl fluorophosphate, 2-amino-2-methylpropanoyl, cytochalasin B, BSA, cytochrome c, peptatin A, and leupeptin (Sigma); superoxide dismutase and FMLP (Calbiochem, La Jolla, CA); PAF (Bachem, Philadelphia, PA); Fura-2 acetoxyethyl ester (Molecular Probes, Eugene, OR); pertussis toxin (List Biological Labs, Campbell, CA); human recombinant GM-CSF (Genzyme, Boston, MA); and [³⁵S]arachidonic acid (American Radiolabeled Chemicals, St. Louis, MO; 55 mCi/mmol). We thank Lilly Research Labs, Indianapolis, IN; Amgen Inc., Thousand Oaks, CA; Dr. James Clark, Genetics Institute, Cambridge, MA; and Dr. Robert Ulevitch, Scripps Research Foundation, La Jolla, CA, for donating, respectively, LY 255283, recombinant human G-CSF, rabbit polyclonal antibody to rat cPLA₂, and rat polyclonal antibody to rabbit antibody to IgG (Transduction Labs, Lexington, KY); 0.45 m nitrocellulose membranes, and ECL kits (Amersham Corp.); PhosphoPlus MAPK antibody kits (New England Biolabs); phenylmethylsulfonyl fluoride, diisopropyl fluorophosphate, 2-amino-2-methylpropanoyl, cytochalasin B, BSA, cytochrome c, peptatin A, and leupeptin (Sigma); superoxide dismutase and FMLP (Calbiochem, La Jolla, CA); PAF (Bachem, Philadelphia, PA); Fura-2 acetoxyethyl ester (Molecular Probes, Eugene, OR); pertussis toxin (List Biological Labs, Campbell, CA); human recombinant GM-CSF (Genzyme, Boston, MA); and [³⁵S]arachidonic acid (American Radiolabeled Chemicals, St. Louis, MO; 55 mCi/mmol). We thank Lilly Research Labs, Indianapolis, IN; Amgen Inc., Thousand Oaks, CA; Dr. James Clark, Genetics Institute, Cambridge, MA; and Dr. Robert Ulevitch, Scripps Research Foundation, La Jolla, CA, for donating, respectively, LY 255283, recombinant human G-CSF, rabbit polyclonal antibody to rat cPLA₂, and rat polyclonal antibody to...
times (10 mM Tris and 0.1% Tween, 100 mM NaCl, pH 7.5), incubated with horseradish peroxidase-linked anti-IgG antibody, and analyzed by ECL. For tyrosine phosphorylated MAPK, samples (4 x 10^6 cells/mg) were resolved by SDS-polyacrylamide gel electrophoresis (10% gel electrophoresis (50 mM APr, run 6 h) and electrotransferred (6 h, 25 MV) to polyvinyl difluoride membranes. The membranes were prepared, blocked, and treated with anti-ERK-1, -2, and -4, and alkaline phosphatase-linked anti-rabbit IgG as recommended by the supplier of the PhosphoPlus antibody kits. For cPLA2, samples (1 x 10^6 cells/mg) were resolved by SDS-polyacrylamide gel electrophoresis (7.5%) gel electrophoresis (22 mM APr, run 5 h to 14 cm) and transferred (60 mAmp, 12 h) to nitrocellulose membranes. Membranes were blocked with 5% milk, washed three times in 0.2% Tween in 50 mM phosphate-buffered normal saline, blotted with anti-cPLA2 antibody (34), incubated with horseradish peroxidase-linked anti-rabbit IgG for 2 h, and analyzed by ECL.

PMN Ca^{2+} Depletion and Pertussis Toxin Treatment—PMN, 6 x 10^6/ml, were incubated at 37°C for 90 min in Ca^{2+}-free Hanks’ buffer containing 1 mM EGTA and 1 mM Fura-2 AM, washed twice, incubated (6 x 10^6 cells/ml) in Hanks’ buffer containing 0 or 1.4 mM Ca^{2+} at 37°C for 20 min and then challenged. These Ca^{2+}-depleted PMN, if incubated with no Ca^{2+}, fail to alter cytosolic Ca^{2+} upon challenge by Ca^{2+}-mobilizing agonists, but if incubated with 1.4 mM Ca^{2+}, mount full Ca^{2+} transient responses (45). For pertussis toxin studies, PMN (1.8 x 10^6/ml) in Ca^{2+}-free buffer were incubated with 4 mM pertussis toxin or 100 mM BSA at 37°C for 120 min, treated with 1.4 mM Ca^{2+} for 20 min, and challenged for 1-5 min. These PMN have 60% lower Ca^{2+} transient and G protein activation responses to 5-HETE, perhaps because of the extensive incubation period (9, 10). Following challenge, PMN suspensions were centrifuged (12,000 g, 5 s; 20°C) to isolate 400 nM Fura-2 AM, washed twice, incubated 20 M Ca^{2+} for 2 min released, respectively, 12 S.E.; 6% net activity released, (n = 6) or superoxide dismutase-inhibitable absorbance in optical density units (O.D.U.) (representative of three studies).

**RESULTS**

Degranulation and O_2 Production—GM-CSF, when incubated with PMN at optimal levels (200 pw, 20 min), increased the degranulation activities of FMLP, PAF, and LT_B2 up to 10-fold. It had even greater effects on 5-oxoETE, 5-oxo-15(0H)-ETE, and 5-HETE, converting these otherwise weak agents to powerful degranulators with respective potencies (LT_B2 = 100) of 20, 10, and 2 (Fig. 1, A and B). Similar results occurred with oxidative metabolism. 5-oxoETE and LT_B2 caused GM-CSF-treated PMN to produce O_2 (Fig. 1C) but only LT_B2 stimulated this response in unprimed cells (data not shown). Hence, 5-oxoOH-ETEs approach LT_B2 in potency on GM-CSF-primed PMN yet, unlike LT_B2, have little activity on unprimed cells. This suggests that 5-oxoOH-ETEs do not utilize LT_B2 receptors and is supported by studies using a LT_B2 receptor antagonist. PMN incubated with 200 pw GM-CSF for 18 min and 0 or 1 uM LY 255283 for 2 min released, respectively, 12 ± 3% (mean net percentage of total cell enzyme released, ± S.E.; n = 6) or 1 ± 2% lysozyme in response to 3 mM LT_B2 and 12 ± 2% or 11 ± 2% in response to 16 mM 5-oxoETE. 15-oxoETE and 15-HETE were inactive with or without GM-CSF. The priming response to GM-CSF required 7.5 min to develop, peaked at 20 min, and increased with increasing concentrations of GM-CSF between 2 and 200 pw. G-CSF primed PMN degranulation responses to FMLP, LT_B2, PAF, 5-oxoETE, 5-oxo-15(0H)-ETE, and 5-HETE (Fig. 1D). Priming in response to G-CSF was more rapid (optimal within 5 min) but led to somewhat less prominent responses than priming by GM-CSF. Thus, 5-oxoOH-ETEs powerfully stimulate PMN primed by hematopoietic cytokines and act through a structurally specific and LT_B2 receptor-independent mechanism.

Secretory Vesicle Mobilization—In gradients of disrupted PMN, surface alkaline phosphatase localized to fractions 4–6 while latent alkaline phosphatase distributed bimodally to fractions 5–7 and 10–13. PMN exposed to 500 mM 5-oxoETE had increased exocytosis of secretory vesicles, as evidenced by raised surface and lowered latent alkaline phosphatase activity (Fig. 2). GM-CSF (200 pw), G-CSF (100 pw), and LT_B2 (1000 pw), but not 500 mM 15-oxoETE, had the same actions. Hence, 5-oxoETE mimics FMLP, PAF, and LT_B2 (42–44) in translocating secretory vesicles to the cell surface. We also examined unfractionated cavitations. In agreement with fractionation studies, cavitations from PMN exposed to GM-CSF, G-CSF, LT_B2, 5-oxoETE, or 5-HETE had low latent (Fig. 3), elevated surface, and unchanged total alkaline phosphate activities (data not shown). The responses to these stimuli were concentration dependent with ED_50 values of 0.02, 1, 4, 20, and 200 nm, respectively. Again, 15-oxoETE (Fig. 3) and 15HETE (data not shown) were inactive and LY 255283 blocked LT_B2 but not 5-oxoETE or 5-HETE (Table I). We conclude that 5-oxoOH-ETEs mobilize secretory vesicles by a structurally specific and LT_B2 receptor-independent mechanism. Priming is not needed for this effect.

MAPKs—Anti-ERK-1 antibody revealed three immunoreactive proteins in PMN, here designated as p40, p42, and p44 according to their electrophoretic mobility (Fig. 4A). To identify these bands further, we used four other antibodies. Polyclonal anti-ERK-1 K23 antibody reacted with all three bands. Monoclonal anti-ERK-2 antibody reacted with p42 exclusively (Fig. 4A). Polyclonal anti-ERK-2 C-14 antibody reacted with p44 and p42 but only to a relatively minor extent with p40 (data not shown). An antibody to p38 identified a single band, which did
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Effects of the LTB₄ receptor antagonist, LY 255283, on the stimulus-induced translocation of secretory vesicles in PMN

PMN were treated with LY 255283 or its vehicle for 2 min, challenged with the indicated stimulus for 20 min, and subjected to N₂ cavitation. Post-nuclear cavitated were assayed for latent alkaline phosphatase activity. * p < 0.05. Students’ paired t-tests above those for PMN that were stimulated in the absence of LY 255283.

| Stimulus       | LY 255283(µM) |
|----------------|---------------|
|                | 0             | 2              |
| BSA            | 28.4 ± 1.3*   | 28.5 ± 3.3    |
| 5-OxoETE, 500 nM | 16.6 ± 1.4    | 15.7 ± 1.9    |
| 5-OxoETE, 50 nM  | 20.9 ± 1.9    | 19.7 ± 1.8    |
| 5-OxoETE, 5 nM  | 22.7 ± 2.0    | 20.6 ± 1.7    |
| 5-HETE, 5,000 nM | 15.4 ± 2.0    | 15.8 ± 2.4    |
| 5-HETE, 500 nM  | 21.1 ± 1.6    | 19.4 ± 3.5    |
| 5-HETE, 50 nM   | 24.6 ± 1.8    | 25.9 ± 3.1    |
| LTB₄, 100 nM    | 14.4 ± 1.5    | 20.4 ± 2.5*   |
| LTB₄, 10 nM     | 15.6 ± 2.2    | 23.2 ± 5.3*   |
| LTB₄, 1 nM      | 20.3 ± 2.4    | 28.5 ± 3.5*   |

* Percentage of total alkaline phosphatase that is latent (n = 5–8 ± S.E.).

Identification of the MAPKs in human PMNs. PMN were stimulated with G-CSF or GM-CSF for 5 min or LTB₄, 15-HETE, or 5-OxoETE for 1 min and lysed. Lysates were resolved by 12% polyacrylamide SDS gel electrophoresis, transferred to nitrocellulose membranes, reacted with the indicated antibody, and visualized (see “Experimental Procedures”). While the stimuli caused p40 and p42 to split, p44 is shown only to broaden. However, analyses using 10% polyacrylamide gels indicated that p44 clearly split into two bands in PMN exposed to 5-OxoETE, LTB₄, G-CSF, or GM-CSF. The results presented are representative of seven experiments.

p40 on exposure to 100 nM 5-OxoETE (Fig. 6). Similar results were observed using anti-ERK-1-Y(P) antibody. Unstimulated PMN did not contain phosphorylated MAPK. However, anti-ERK-1-Y(P) reactive bands (p40, p42, and p44) were observed at 0.25 and 1 min after stimulation (Fig. 7). The reactive bands were transient and were not observed at 4 min. PMN, primed with either 20 or 200 pM GM-CSF for 20 min, showed greatly enhanced reactivity in all three bands upon challenge with 5-OxoETE at all times tested (Fig. 7).

cPLA₂—In resting PMN, antibody to cPLA₂ detected a protein at ~85 kDa plus a variable amount of a slower migrating species. Since these two bands correspond to the non-activated and activated forms of cPLA₂ (43, 48), some cPLA₂ may have been activated by our cell manipulations. In any case, resting PMN had by far the most dominant form of cPLA₂ in the faster

not comigrate with p40. The band reacting with p38 antibody did not show a mobility shift in cells stimulated with 5-OxoETE, FMLP, or other stimuli under conditions were all three ERK-1 antibody-reactive bands exhibited mobility shifts (see below). Based on the specificities of these antibodies (47–54), these data identify p44 and p42 as ERK-1 and -2, respectively, and p40 as an ERK-1-like protein. We found that our ability to detect p40 was lost very quickly (3–5 min) if the samples were left in the solution of protease and phosphatase inhibitors without boiling. This may explain why previous investigators have not observed this protein. All three proteins were converted to slower migrating forms in PMN stimulated by G-CSF, GM-CSF, LTB₄, or 5-OxoOH-ETEs but not by cells incubated with 15-HETE or 15-oxoETE (Fig. 4, A and B). Such mobility shifts characteristically occur when MAPKs are activated by phosphorylation (46, 47). Indeed, we observed that PMN stimulated with 5-OxoETE showed new bands at p44, p42, and p40 that were detected with anti-ERK-1-Y(P) antibody (Fig. 7). MAPK gel-shift responses to GM-CSF started at 5 min, peaked by 20 min, and slowly declined while those to 5-OxoETE and LTB₄ began by 0.25 min, maximized at 1 min, and disappeared in less that 5 min (Fig. 5A). In studies not shown, LY 255283 (1–4 µM) blocked responses to 0.1–10 nM LTB₄, yet did not alter those to 5–500 nM 5-OxoETE. Finally, no stimulus, even at levels 50-fold higher than those causing half-optimal effects (ED₅₀ levels were 5–8 nM), lowered latent alkaline phosphatase; 5-HETE also lowered latent alkaline phosphatase; 15-HETE, LTB₄, or 5-OxoETE were inactive.

Primed PMN achieved far greater responses. PMN treated with 200 or 20 pM GM-CSF phosphorylated essentially all of the p42 and p44, and shifted the p40 on exposure to 100 nM 5-OxoETE (Fig. 6). Similar results were observed using anti-ERK-1-Y(P) antibody. Unstimulated PMN did not contain phosphorylated MAPK. However, anti-ERK-1-Y(P) reactive bands (p40, p42, and p44) were observed at 0.25 and 1 min after stimulation (Fig. 7). The reactive bands were transient and were not observed at 4 min. PMN, primed with either 20 or 200 pM GM-CSF for 20 min, showed greatly enhanced reactivity in all three bands upon challenge with 5-OxoETE at all times tested (Fig. 7).

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2 A. B. Nixon, unpublished data.
migrating form and up to 100% of this was phosphorylated during PMN challenge with each stimulus studied (Fig. 5B). cPLA2 responses began with, but greatly outlasted, those of MAPK (Fig. 5B). GM-CSF, G-CSF, LTB4, 5-oxoETE, 5-oxo-15(OH)-ETE, and 5-HETE produced half-maximal responses at 0.02, 1, 3, 20, and 100 nM, respectively, while 15-HETE and 15-oxoETE were inactive at levels up to 5,000 nM (data not shown). Finally, LY 255283 blocked LTB4 but not 5-oxoETE from activating cPLA2 (data not shown). We conclude that PMN exposed to diverse stimuli activate cPLA2 as well as p44, p42, and p40 MAPK proteins. 5-Oxo/OH-ETEs induce these changes by acting through a structurally specific, LTB4 receptor-independent mechanism. This response is enhanced by pretreatment with GM-CSF.

Arachidonic Acid Release—PMN incubated with [14C]arachidonic acid incorporated > 90% of added radioactivity. When the prelabeled PMN’s were incubated with BSA ± GM-CSF there was no increase in [14C]arachidonic acid release compared to control cells (~1% of total cell label). PMN exposed to ≥ 500 nM 5-oxoETE released small amounts of radiolabel. GM-CSF (≥ 20 pM), while not altering basal arachidonic acid release, greatly increased the potency of 5-oxoETE in eliciting this response (Fig. 8). In the presence of 2000 pM GM-CSF, 5 nM 5-oxoETE was a thousandfold more active (Fig. 8). By comparison, 500 and 5000 nM 15-HETE were totally ineffective in stimulating arachidonic acid radiolabel release in unprimed or GM-CSF (20–2000 pM)-primed PMN (data not shown). The arachidonic acid release in response to FMLP (1000 nM) was also enhanced by pretreatment with GM-CSF. Hence, the positive cooperation between 5-oxoETE and GM-CSF in activating MAPK (Figs. 6 and 7) was associated with a similar co-operativity in mobilizing arachidonic acid (Fig. 8).

Ca2+ and Pertussis Toxin Studies—PMN depleted of Ca2+ was a thousandfold more active (Fig. 8). By comparison, 500 and 5000 nM 15-HETE were totally ineffective in stimulating arachidonic acid radiolabel release in unprimed or GM-CSF (20–2000 pM)-primed PMN (data not shown). The arachidonic acid release in response to FMLP (1000 nM) was also enhanced by pretreatment with GM-CSF. Hence, the positive cooperation between 5-oxoETE and GM-CSF in activating MAPK (Figs. 6 and 7) was associated with a similar co-operativity in mobilizing arachidonic acid (Fig. 8).
and resuspended in Ca²⁺-free buffer had normal MAPK mobility shifts in response to 5-oxoETE and LTβ4 as compared to control cells (data not shown) or Ca²⁺-depleted PMN suspended with 1.4 mM Ca²⁺ (Fig. 9A). On the other hand, pertussis toxin-treated PMN had reduced MAPK responses to 5-oxoETE and LTβ4 (Fig. 9B). We also used the anti-ERK-1 γPO₄ antibody to confirm that extracellular Ca²⁺ upon stimulation but when incubated with 1.4 mM Ca²⁺ had full Ca²⁺-transient responses. Panel B, PMN were treated with 0 or 1.4 μM pertussis toxin and challenged with 5-oxoETE or LTβ4. The results presented are representative of four studies.

**DISCUSSION**

GM-CSF and G-CSF made PMN as much as 10-fold more sensitive to LTβ4, PAF, and FMLP. They had an even greater impact on 5-oxoOH-ETEs and LTβ4 on MAPK require pertussis toxin-sensitive G proteins. Hence, the effects of 5-oxoETE and LTβ4 (Fig. 9A) had full Ca²⁺-transient responses. Panel B, PMN were treated with 0 or 1.4 μM pertussis toxin and challenged with 5-oxoETE or LTβ4. The results presented are representative of four studies.

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results with GM-CSF and extend them to LTβ4. Both stimuli caused mobility shifts in all three proteins detected by anti-ERK-1 antibody. Responses to GM-CSF developed slowly and endured for >40 min. The response to LTβ4 peaked at 1 min and ended by 5 min. G-CSF also induced p44ERK-1, p42ERK-2, and p40 mobility shifts, although its effects were modest. This agrees with findings that G-CSF weakly stimulates PMN to increase MAPK phosphorylating activity and to tyrosine phosphorylate 40–44-kDa bands (33, 60). We further found that 5-oxo/OH-ETEs caused rapid (0.25 min) and transient (2.5 min) mobility shifts in p44ERK-1, p42ERK-2, and p40 (Fig. 5). Hence, activation of all three proteins seems a constant and possibly central event in PMN activation.

The data also indicate that p40 is an extracellular signal-regulated protein, possibly related to the 36–40-kDa proteins tyrosine-phosphorylated in PMN exposed to GM-CSF or tumor necrosis factor-α (58–60). Further studies will be required to determine if p40 is a new MAPK, or a splicing or post-translationally modified variant of ERK-1 or related to JNks (53–55). However, the p40 detected in PMN reacted with a series of antibodies that are directed toward peptide sequences that are not found in JNks or p38 (56, 62) and it seems unlikely that the p40ERK-1-like protein seen here is related to the latter kinases.

cPLA2 mobility shifts (Fig. 5B) correlated closely with changes in MAPK mobility although the former persisted much longer than the latter. MAPKs phosphorylate cPLA2 on serine 505, thereby slowing its migration and doubling its activity (48). In separate work (61), we determined that LTβ4, 5-oxoETE, or 5-HETE each stimulated cPLA2 activity and concomitant phosphorylation of the enzyme. Together, these results make it clear that LTβ4 and 5-oxo/OH-ETEs cause PMN to phosphorylate and activate MAPKs, one or more of which then phosphorylate and activate cPLA2. It is worth emphasizing that such events may be necessary but are not sufficient for many PMN functions. LTβ4 and 5-oxoETE have quite similar effects on MAPK and cPLA2 yet very different impacts on degranulation and O2 production. Clearly, signals other than those of the MAPK and cPLA2 pathways may make critical contributions not only to PMN function but also phospholipid deacylation, arachidonic acid release, and PAF synthesis. It is worth noting that >500 nM 5-oxoETE stimulated small amounts of arachidonic acid release, this effect being comparable to that achieved by 1 μM FMLP, and that GM-CSF enhanced the arachidonic acid release in response to 5-oxoETE (Fig. 8). Hence, the cytokine-primed PMN have not only an increased MAPK activation (Figs. 6 and 7) but also an enhanced arachidonic acid release in response to 5-oxoETE. This suggests that priming occurs at a step before MAPK activation and stimulates cPLA2 activation and arachidonic acid release. In primed PMN, 5-oxoETE may prove to be similarly potent in influencing PAF synthesis as well as other downstream events regulated by MAPKs.

In conclusion, we stress four findings. First, structure-activity relations for 5-oxoETE, 5-oxo-15(OH)-ETE, 5-HETE, 15-PAF synthesis as well as other downstream events regulated by PMN, 5-oxoETE may prove to be similarly potent in influencing PAF synthesis as well as other downstream events regulated by PMKs. Second, per-tussis toxin blocked MAPK responses to LTβ4 and 5-oxo/OH-ETEs, whereas LY 255283 inhibited diverse responses to LTβ4 without influencing those to 5-oxo/OH-ETEs (Fig. 7, Table I). The 5-oxo/OH-ETE recognition system likely involves a G protein- and Ca2+-linked receptor distinct from LTβ4 receptors. Third, Ca2+-depleted PMN responded to 5-oxo/OH-ETEs with MAPK mobility shifts (Fig. 9). These data are the first to indicate that 5-oxoOH-ETEs act on Ca2+-dependent as well as Ca2+-independent pathways, both of which are initiated by G proteins. Fourth, GM-CSF greatly enhanced the effects of 5-oxo/OH-ETEs on degranulation, O2 production, and MAPK activation and arachidonic acid release (Figs. 1 and 6–8). 5-oxoOH-ETEs thus co-operate with hematopoietic cytokines to stimulate MAPK, cPLA2, and functional responses.

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