A Novel Glutathione Containing Eicosanoid (FOG₇)
Chemotactic for Human Granulocytes

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Running Title: Chemotactic Eicosanoid-Glutathione Conjugate
Abstract

A biologically active glutathione adduct of the eicosanoid 5-oxo-eicosatetraenoic acid has been observed as a product formed within the murine peritoneal macrophage. This five-oxo glutathione adduct (FOG₇) was structurally characterized using electrospray tandem mass spectrometry as a 1,4 Michael addition product 5-oxo-7-glutathionyl-8,11,14-eicosatrienoic acid. FOG₇ was found to be highly potent in stimulating eosinophils as well as neutrophil chemotaxis, also capable of initiating actin polymerization, without elevating intracellular free calcium ion concentration within either the eosinophil or polymorphonuclear leukocyte. These biological responses suggest that either FOG₇ activates a subset of receptors mediating the broader biological activity of the parent eicosanoid 5-oxo-ETE or that a receptor not activated by 5-oxo-ETE participates in the chemotactic activity of FOG₇. The only other known biologically active glutathione adduct has been leukotriene C₄, another eicosanoid which exerts potent effects through the cys-LT receptor. The biochemical parallel between the formation of LTC₄ and FOG₇ suggests an interesting mechanism by which biologically active eicosanoids derived from electrophilic intermediates may have unique distribution and prolonged efficacy in vivo.
The enzymatic oxidation of arachidonic acid leads to the formation of a diverse family of biologically active eicosanoids that play important roles as intracellular chemical communicators of cellular activation. Oxidation of arachidonic acid catalyzed by 5-lipoxygenase leads to initial formation of 5-hydroperoxyeicosatetraenoic acid (5-HpETE) (1,2) and the chemically reactive intermediate leukotriene A\(_4\) (LTA\(_4\)) derived from a second 5-lipoxygenase-mediated reaction using 5-HpETE as substrate. LTA\(_4\) is transformed either into the neutrophil chemotactic leukotriene B\(_4\) (LTB\(_4\)) (3,4) or the glutathione adduct, leukotriene C\(_4\) (LTC\(_4\)), originally called slow reacting substance of anaphylaxis (5). While considerable interest has focused attention on the leukotriene pathway of arachidonate metabolism within cells, the initial 5-HpETE intermediate can also be converted into 5-HETE and further metabolized into 5-oxo-ETE by an NADP\(^+\)-dependent dehydrogenase (6,7). The discovery that 5-oxo-ETE is chemotactic for eosinophils, and to a lesser extent to the human polymorphonuclear leukocyte, has raised interest in this eicosanoid because of the suggested role of eosinophils in diseases such as asthma (8,9). This molecule has additional biological activities on granulocytes such as calcium mobilization (10), actin polymerization (11), and integrin expression (12). Synthesis of 5-oxo-ETE has been demonstrated in neutrophils (13) and monocytes (14). Since termination of biological activity of all eicosanoids is largely a result of metabolic conversion to inactive products, an understanding of metabolic pathways within biosynthetic cells and tissues of origin has been of interest. Previous metabolic studies of 5-oxo-ETE have suggested that cytochrome-P450 dependent \(\omega\)-oxidation in the neutrophil (15) and \(\Delta^6\)-reduction (10) leads to the formation of biologically inactive products. We describe here that an alternative major route of 5-oxo-ETE biotransformation exists within a tissue derived macrophage that results in a profoundly chemotactic eicosanoid.

**Materials and Methods**

**Materials.** 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE) and \([5,6,8,9,11,12,14,15-^2\text{H}_8]\)5-hydroxyeicosatetraenoic acid (D\(_8\)-5-HETE) were purchased from Cayman Chemical Company (Ann Arbor, MI). \([^3\text{H}]\)5-HETE (specific activity 58 Ci/mmol) was purchased from New England Nuclear. \([6,8,9,11,12,14,15-^2\text{H}_7]\) and \([6,8,9,11,12,14,15-^3\text{H}_7]\)5-oxo-ETE were prepared from either D\(_8\)-5-HETE (40 \(\mu\)g) or \([^3\text{H}]\)5-HETE (40 \(\mu\)g cold and 10 \(\mu\)Ci radiolabeled tracer) using 2,3-dichloro-5,6-diaz-1,4-
benzoquinone (Aldrich Chemical Co, Milwaukee, WI) as previously described (16) to afford the corresponding isotope labeled 5-oxo-ETE following purification by RP-HPLC.

Hank's balanced salt solution was purchased from Gibco BRL Life Technologies (Gaithersburg, MD). Indo-1/AM was obtained from Calbiochem (La Jolla, CA). NBD-phallicidin (N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phallacidin) was obtained from Molecular Probes (Eugene, OR). All solvents were HPLC grade and obtained from Fisher Scientific (Fair Lawn, NJ).

**Incubation of peritoneal macrophages and metabolite isolation.** Elicited peritoneal macrophages were obtained from the peritoneal cavity of ICR mice following injection of 1 mL of thioglycolate (10%) into the peritoneum three days prior and cells isolated as previously described (17). After adherence of cells to plastic plates, the cells were gently suspended in 3 mL Hank's balanced salt solution (1.3 x 10^7 cells). 5-Oxo-ETE, [2H7]5-oxo-ETE and [3H7]5-oxo-ETE (0.6 μCi) were incubated with the cells at a concentration of 14 μM and with a D0/D7 ratio of the unlabeled substrate to stable isotope of 2:1. Incubation was carried out at 37°C for 3 hr followed by centrifugation and separation of metabolites present in the supernatant using solid phase extraction technique (18).

Reversed phase HPLC of the solid phase extracted metabolites was carried out using mobile phase A containing 8.3 mM acetic acid buffered to pH 5.7 with NH₄OH and mobile phase B composed of acetonitrile:methanol (65:35, v/v) and a Columbus 5 μ C18 column 150 x 2.0 mm (Phenomenex, Rancho Pales Verdes, CA). HPLC effluent was monitored using a photodiode array detector and fractions collected at 1 min intervals from the column eluted at 200 μL/min with a linear gradient from 15% B to 55% B in 10 min to 80% B in 25 min to 100% B in 30 min and then held at 100% B for a further 5 min.

**Eosinophil and neutrophil chemotaxis.** Chemotaxis of neutrophils and eosinophils were carried out in a Zigmond Chamber (19). Briefly, cells were adhered to a glass slide, inverted over a bridge between two wells where the concentration gradient was formed. The movement of cells were monitored under a microscope with TV video taping when a field contained 7-9 cells. Analysis of direction and velocity graphically assessed at 3 min intervals for 30 min. The test solution containing either buffer or 5-oxo-ETE, FOGγ, and fMLP in one well of the chamber at the concentrations indicated and 6-7
individual cells were monitored. Selection of cells for vector analysis only required that the cells remain in the field of view for the first 10 min of videotaping.

**Actin polymerization and intracellular \([\text{Ca}^{++}]\) determination.** Actin polymerization was assessed by flow cytometry as previously described (20). Neutrophils or eosinophils were incubated with the test compound for 30 sec, cells were then fixed with lysoformalin for 5 min at 37°C. NBD-phallicidin was added to the fixed cells for 10 min at 37°C prior to flow cytometry. A control experiment was run without test substance present for background fluorescence binding.

Intracellular cytosolic calcium was assessed by incubation of neutrophils (10⁷ cells/mL) loaded with the acetoxy methyl ester of Indo-1 as previously described (21). Before the addition of each test substance, CaCl₂ and MgCl₂ were added to the cell suspensions at 1 mM final concentration to 3 x 10⁸ neutrophils in a 4 mL cuvet. The \(K_d\) of 250 nM for the Indo-1 Ca²⁺ complex was used to calculate the intracellular calcium concentration and a \(F_{\text{max}}\) determined by the addition of digitonin at 0.1% and \(F_{\text{min}}\) was determined by the addition of 7.8 mM EGTA in Tris buffer.

**Chemical synthesis of 5-oxo-ETE/GSH adduct.** Addition of glutathione (2 mM) to 5-oxo-ETE (10 μM) was carried out at pH 9.5 aqueous buffer for 4 hr at 37°C. Reversed phase HPLC purification and LC/MS and LC/MS/MS analysis was carried out using identical conditions described above. The most abundant eicosanoid backbone cleavage ion occurred at \(m/z\) 348 corresponding to cleavage between carbon atoms 9 and 10. This 1,6 Michael addition product also had a UV spectrum with absorption maximum at 262 nm consistent with \(\alpha/\beta\)-unsaturated ketone (22).

**Mass spectrometry.** Direct liquid chromatography/mass spectrometry was carried out on a Sciex API-III* triple quadrupole mass spectrometer (PE-Sciex, Thornhill, Ontario, Canada). A 1.0 mm x 150 mm Ultramex-3 C18 reversed phase HPLC column (Phenomenex) was used with the same gradient solvent system for the metabolite separation, but at a flow rate of 50 μL/min. A mass range of \(m/z\) 172 to 700 was scanned at the rate of 3 sec/scan with a spray voltage of -2800 volts and orifice voltage maintained at -50 volts with a collisional offset potential of 15 eV. Collision induced decomposition (CID) were performed with a collision gas thickness (argon) of 150 x 10¹³ molecules/cm².
Results and Discussion

To learn more about the metabolism of 5-oxo-ETE in tissues, murine peritoneal macrophages were incubated with 5-oxo-ETE containing both radiolabeled and stable isotope labeled tracers. Following incubation at 37°C for 3 hr, metabolites were separated by reversed phase HPLC and fractions collected to assess elution of radiolabeled metabolites. Several metabolites were observed (Figure 1A) including several minor metabolites which retained UV chromophores. The structures of these metabolites will be described separately. However, the most abundant metabolite did not absorb light in the UV region and yielded a molecular anion [M-H]⁻ at m/z 624 by negative ion electrospray LC/MS. Metabolism of the stable isotope labeled D₇-5-oxo-ETE yielded a corresponding [M-H]⁻ anion at m/z 630 indicating the retention of only six deuterium atoms, suggesting loss of a single deuterium atom during the biochemical conversion. Collisional activation of metabolite A following negative ion electrospray ionization yielded an abundant product ion (Figure 1A, inset) at m/z 306 consistent with the addition of glutathione to 5-oxo-ETE by a Michael addition reaction (18) (22,23).

In order to assess the position of glutathione covalent attachment on the 5-oxo-ETE backbone, this metabolite was analyzed by positive ion electrospray ionization mass spectrometry using an ion trap mass spectrometer. Collisional activation of the [M+H]⁺ cation at m/z 626 yielded an abundant product ion at m/z 497 which corresponded to the loss of the γ-glutamyl portion of the glutathione adduct. This product ion has been observed in the collisional activation of other glutathione adducts of arachidonic acid. Subsequent collisional activation of m/z 497, which was possible in the ion trap mass spectrometer as an MS³ experiment, yielded an exceptionally rich product ion spectra as shown in Figure 1B. Abundant ions were observed corresponding to cleavage of the carbon-sulfur bond with product ions m/z 319 and 179 consistent with a cysteiny1 glycine adduct. The product ion at m/z 367 was particularly relevant since it corresponded precisely to cleavage of the arachidonate carbon backbone between carbon-6 and -7. This fragmentation would be expected if a carbon-sulfur thioether bond was formed at carbon-7 which would facilitate formation of a delocalized carbonium ion and loss of the first six carbon atoms of arachidonic acid. This MS³ collisional spectrum was consistent with the addition of glutathione to 5-oxo-ETE in a 1,4 Michael addition reaction. Comparison of chromatographic and mass spectral data of this metabolite with the nonenzymatically synthesized 1,6 Michael addition product of glutathione to 5-oxo-
ETE as well as the isobaric eicosanoid, LTC$_4$, clearly revealed that the 5-oxo-ETE metabolite was a novel glutathione adduct of 5-oxo-ETE. Thus, the most abundant metabolite of 5-oxo-ETE made by the murine peritoneal macrophage corresponded to the compound 5-oxo-7-glutathionyl-8,11,14-eicosatrienoic acid which we abbreviate as FOG$_7$ (Scheme 1).

- insert Scheme 1 here -

A curious feature of this 5-oxo-ETE metabolite was that it is isobaric to LTC$_4$, both having a molecular weight of 625 daltons. In general, the glutathione conjugation of reactive chemical entities such as dieneones is a mechanism to reduce the potential cellular toxicity of such agents (24). However, in the case of LTA$_4$, this leads to the formation as a potent biologically active substance LTC$_4$ (5). It was therefore of interest to investigate whether or not this novel glutathione adduct of 5-oxo-ETE was also biologically active. The ability of the metabolite to induce neutrophil and eosinophil chemotaxis was tested using a Ziangmond chamber that could assess both chemotaxis and chemokineses over a concentration gradient (19). When eosinophils were present in the chemotactic chamber (Figure 2A), both 5-oxo-ETE and FOG$_7$ at concentrations of 10 nM and 100 nM induced cellular movement towards each respective chemotactic agent as compared to buffer control. Neutrophils were also found to be chemoattractive for FOG$_7$ at 10 nM, similar to that observed for 5-oxo-ETE (Figure 2B). Calculation of the velocity of cellular migration (Table 1) revealed that FOG$_7$ was more potent than 5-oxo-ETE in chemotactic velocity. Significantly, FOG$_7$ retained the chemotactic and chemokinetic activities of the metabolic precursor 5-oxo-ETE, revealing that processing by this pathway did not lead to inactivation of chemotactic properties of 5-oxo-ETE.

The biological activity of FOG$_7$ was also compared to 5-oxo-ETE and fMLP for inducing actin polymerization in the human polymorphonuclear leukocyte. Using NBD-phallicidin to assess formation of F-actin (20), eosinophils or neutrophils were incubated for 30 sec following fixation and analyzed by flow cytometry. Actin polymerization in neutrophils was induced by fMLP as expected. 5-Oxo-ETE as well as FOG$_7$ also elicited a significant shift in fluorescence, indicating the rapid formation of F-actin (Figure 3). Eosinophils yielded similar results (data not shown). These data supported the chemotaxis data by showing that FOG$_7$ was able to polymerize actin in both neutrophils and eosinophils.
Another reported activity of 5-oxo-ETE was the elevation of intracellular calcium in both neutrophils or eosinophils as assessed using Indo-1AM fluorescence (21). As previously shown, 5-oxo-ETE (10 nM) (12) as well as fMLP (10 nM) (25) induced in a dose dependent manner, a significant elevation of intracellular calcium ion in the human neutrophil (Figure 4). However, FOG7 did not induce calcium mobilization in the neutrophil, even at concentrations as high as 320 nM. A similar lack of response was observed for eosinophils loaded with Indo-1AM. The 5-oxo-ETE glutathione adduct was thus not effective in elevating intracellular calcium ion as opposed to the parent 5-oxo-ETE. Since 5-oxo-ETE may activate more than one receptor (11,26,27), it is possible that FOG7 could signal only through a subset of these receptors. More likely, however, these two eicosanoids may exert their effects through separate receptors.

Together, these results indicated that a major route of 5-oxo-ETE processing in the peritoneal macrophage was through conjugation with glutathione in a 1,4 Michael addition reaction even though the 1,6-adduct was the more likely product. While this metabolic process may reduce the chemical reactivity of 5-oxo-ETE, it nonetheless did not terminate the biological activity of this eicosanoid. Rather the glutathione adduct, FOG7 was profoundly active in eliciting chemotaxis and chemokinesis of both the human neutrophil and eosinophil. There is, furthermore, a striking biochemical parallel in the formation of this glutathione adduct of 5-oxo-ETE and the glutathione adduct of LTA4 termed LTC4. Both conjugation reactions result in the formation of the only known glutathione adducts which are biologically active. Conjugation of glutathione profoundly alters both chemical and biochemical properties of precursor eicosanoids as well as subsequent metabolic decomposition reactions. This is particularly true in chemical partition properties of these glutathione adducts. Both precursor eicosanoids, LTA4 and 5-oxo-ETE, are highly lipophilic and likely have difficulty in exiting synthetic cells without the assistance of carrier protein substances to promote movement from the lipid-membrane environment. The attachment of a hydrophilic tripeptide (glutathione) to these substances alters this substantially. In this respect, glutathione conjugation of a reactive eicosanoid intermediate, as exemplified by LTC4 and FOG7 may be a mechanism by which cells can alter distribution of biologically active eicosanoids, direct their actions to specific tissue targets, and possibly prolong efficacy in vivo.
Acknowledgments

This work was supported, in part, by a grant from the National Institutes of Health (HL25785).

REFERENCES


Table 1 Chemotaxis parameters\(^1\) for human eosinophils and human neutrophils exposed to 5-oxo-ETE, FOG\(_7\), and fMLP in a Zigmond Chamber (19).

<table>
<thead>
<tr>
<th></th>
<th>(\mu/min)</th>
<th>(V_c^2)</th>
<th>(V_d)</th>
<th>(V_{act})</th>
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<td><strong>Eosinophils</strong></td>
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<tr>
<td>5-oxo-ETE 10 nM</td>
<td></td>
<td>4.6 ± 1.5(^3)</td>
<td>10.4 ± 0.8</td>
<td>12.9 ± 1.2</td>
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<td></td>
<td>11.5 ± 1.5</td>
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<td>FOG(_7) 10 nM</td>
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<td>14.3 ± 0.2</td>
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<td>12.9 ± 2.4</td>
<td>20.0 ± 1.6</td>
<td>24.9 ± 1.8</td>
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<td><strong>Neutrophils</strong></td>
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<td></td>
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<tr>
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<td>4.4 ± 0.8</td>
<td>7.2 ± 1.3</td>
<td>9.6 ± 1.7</td>
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<td>18.2 ± 1.7</td>
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<tr>
<td>FOG(_7) 10 nM</td>
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<td>17.3 ± 0.6</td>
<td>21.5 ± 6.8</td>
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<tr>
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<td>12.8 ± 1.7</td>
<td>16.9 ± 2.3</td>
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<td>fMLP 1 (\mu)M</td>
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<td>6.8 ± 0.2</td>
<td>8.8 ± 0.3</td>
</tr>
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</table>

\(^1\)Velocity determined by videography as microns of cell movement per minute.

\(^2\)\(V_c\) is the vectored velocity towards the test substance chamber, \(V_d\) is the displacement velocity, and \(V_{act}\) is the total distance moved per min.

\(^3\)Average and standard error of the mean (\(n = 6\) or 7 individual cells).
**Figure Legends**

**Figure 1** Purification and mass spectrometric analysis of metabolite termed FOG₇. (A) Reversed phase HPLC separation of 5-oxo-ETE metabolites obtained following incubation of 5-oxo-ETE (14 μM) with 1.3 x 10^7 cells followed by solid phase extraction and gradient elution. Individual fractions were collected for determination of radioactivity content using scintillation counting. Inset shows the tandem mass spectrum (MS/MS) of the abundant radioactive component present in the fraction eluting at 13 min yielding an abundant negative ion at m/z 624. The major product ion at m/z 306 formed by the loss of 308 u corresponds to the loss of glutathione. (B) MS/MS/MS (MS³) analysis of the component in fraction 13, having an abundant positive ion [M+H]^+ at m/z 626 which was collisionally activated to yield the product ion for m/z 497 and subsequent collisional activation of this product ion to yield the mass spectrum indicated. The structure of the proposed product ion m/z 497 is illustrated with abundant ions corresponding to specific cleavage reactions indicated. The ion at m/z 367 supported the carbon sulfur bond at carbon-7 of the original 5-oxo-ETE substrate.

**Figure 2** Vector analysis of human peripheral eosinophils (n=4-7) measured in a Zigmond chamber as recorded under microscopic investigation. The orientation of the cells were similar for all panels where the test solution containing the indicated compound is the top of the graph whereas the corresponding buffer is in the well corresponding to the bottom of the graph. Measurement of the migration of each eosinophil was carried out at timed intervals (7 min) where each cell is normalized to a single origin.

**Figure 3** Measurement of F-actin in human eosinophils using flow cytometry and NBD-phallicidin as previously described (20). Fluorescence of control cells is indicated by the blue trace while cells treated with 5-oxo-ETE, FOG₇ or fMLP are indicated by red, green, and orange trace, respectively. The concentration of 5-oxo-ETE was experimentally determined using the ultraviolet absorption determination of standard solutions while FOG₇ was determined by quantitative mass spectrometry with a purified sample of FOG₇ derived from radiolabeled 5-oxo-ETE of known specific activity.

**Figure 4** Response of human neutrophils preloaded with Indo-1/AM following addition of 5-oxo-ETE (□) and FOG₇ (♦) in both eosinophils and neutrophils. The increased fluorescence was converted to concentration of calcium as previously described (21).
Bowers_Figure 1
Bowers_Figure 2
Bowers _Figure 3_
Bowers_Figure 4
Scheme 1
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