Enhancement of Chemotactic Factor-Stimulated Neutrophil Oxidative Metabolism by Leukotriene B₄

By James C. Gay, Jeffrey K. Beckman, Alan A. Brash, John A. Oates, and John N. Lukens

Leukotriene B₄ (LTB₄) is a potent primary stimulator of neutrophil chemotaxis, aggregation, and degranulation and induces superoxide production at higher concentrations. In order to determine whether LTB₄ modulates neutrophil responses to oxidative stimuli, human neutrophils (PMNs) were incubated with LTB₄ prior to stimulation with f-Met-Leu-Phe (fMLP, 10⁻⁹ mol/L), opsonized zymosan (OZ, 250 μg/mL), or phorbol myristate acetate (PMA, 32 nmol/mL). Superoxide (O₂) production by stimulated PMNs was assessed by the superoxide dismutase-inhibitable reduction of cytochrome c. LTB₄ alone did not stimulate O₂ production in concentrations below 10⁻⁷ mol/L and had no effect on the O₂ assay. In the concentration range of 10⁻¹² to 10⁻⁸ mol/L, LTB₄ did not alter O₂ release induced by OZ or PMA. In contrast, LTB₄-treated cells demonstrated enhanced O₂ production following exposure to fMLP, and in the presence of 10 nmol/L LTB₄, generated 180% ± 41% of O₂ quantities produced by control cells (n = 23). Enhancement was LTB₄ dose-dependent, was maximal in the range of 1 to 10 nmol/L LTB₄, was not reversed by removal of the lipid from the medium prior to fMLP stimulation, and was not dependent on the presence of Ca²⁺ or Mg²⁺ in the suspending medium. Chemiluminescence of fMLP-stimulated neutrophils was increased to 323% of controls in neutrophils preincubated with 10 nmol/L LTB₄. Unlike augmentation of oxidative responses to fMLP seen with other degranulating stimuli, enhancement by LTB₄ was not correlated with an increase in ³H-fMLP receptor binding. These results indicate that, in addition to its primary effects on neutrophil function, LTB₄ modulates PMN oxidative responses to the chemotactic peptide and, thus, may amplify the release of oxygen metabolites at inflammatory foci.

PRODUCTS OF ARACHIDONIC ACID lipoygenation are important mediators of inflammation, and several of these compounds directly stimulate neutrophil responses. The arachidonate metabolite that appears to have the greatest direct effect on neutrophil function is 5(S), 12(R)-dihydroxy-eicosatetraenoic acid, or leukotriene B₄ (LTB₄). This lipid induces neutrophil aggregation and degranulation, but is much more effective as a chemoattractant, having chemotactic potency equal to synthetic formylated peptides and C5a. Previous reports from our laboratory and others have shown that some chemotactic factors regulate neutrophil functions. For instance, neutrophils exposed to low concentrations of N-formyl-methionyl-leucyl-phenylalanine (fMLP) or C5a exhibited enhanced superoxide anion production in response to the nonchemotactic stimuli phorbol myristate acetate (PMA) and fluoride ion. In contrast, the oxidative responsiveness of cells incubated with chemotactic factors was diminished upon subsequent challenge with the chemotactic peptides. Because LTB₄ shares several neutrophil-stimulating properties with peptide chemoattractants, the present study was undertaken to investigate the ability of LTB₄ to modify neutrophil oxidative responses. The results demonstrate that preexposure to LTB₄ enhances neutrophil oxidative responsiveness to the chemotactic peptide. This regulatory effect of the leukotriene could lead to amplification of neutrophil release of oxygen metabolites at inflammatory foci.

MATERIALS AND METHODS

Ferricytochrome c (type III), zymosan, dimethyl sulfoxide (DMSO), fMLP, Ficoll-Hypaque (Histopaque), superoxide dismutase (SOD), phorbol myristate acetate (PMA), xanthine, xanthine oxidase, and bovine serum albumin (BSA) were purchased from Sigma Chemical Company, St Louis. Hanks' balanced salt solution (HBSS, with or without calcium and magnesium) was obtained from Grand Island Biological Company (GIBCO), Grand Island, NY. Tritiated fMLP (³H-fMLP, 48.3 Ci/mmol) was purchased from New England Nuclear, Boston. Dibutyl phthalate was obtained from Aldrich Chemical Company, Milwaukee, and Apiezon C oil was supplied by Apiezon Products Ltd, London. Leukotriene B₄ (LTB₄) was kindly provided by Dr J. Rokach, Merck Frosst Canada, Inc, Quebec.

fMLP and PMA were dissolved in DMSO at a concentration of 10 mg/mL. These were further diluted in HBSS prior to use. Cytochrome c was dissolved in HBSS at a concentration of 1.0 mmol/L (pH adjusted to 7.4 with dilute HCl). SOD was dissolved in HBSS at a concentration of 1 mg/mL.

Zymosan was opsonized by incubation in fresh normal serum (10 mg/mL) at 37°C for 30 minutes. The particles were then washed by centrifugation and resuspended in HBSS at a concentration of 5 mg/mL.

LTB₄ was stored at −70°C in methanol at 100 μg/mL. At the time of usage in neutrophil assays, an aliquot from the stock solution was evaporated under N₂ and redissolved in bovine serum albumin (BSA, 50 mg/mL) to provide the concentration indicated in the final incubation medium.

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Supported in part by National Institute of Allergy and Infectious Diseases grant AI-06949 and National Institutes of Health grant GM 15431.

Submitted Feb 17, 1984; accepted April 16, 1984.

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Neutrophil Suspensions

Human neutrophils were isolated from heparinized venous blood from healthy adult volunteers. Isolation was initiated by enhanced erythrocyte sedimentation, followed by layering of plasma and buffy coats onto Ficoll-Hypaque gradients. The gradients were centrifuged at 1,200 rpm for 30 minutes, and erythrocytes remaining in the cell layers were lysed with 0.15M NH₄Cl. The resulting neutrophils were washed and resuspended in HBSS. This isolation procedure consistently yielded neutrophils with greater than 98% purity and viability.

Neutrophil Superoxide Release

Superoxide (O₂-) was assayed by measuring the O₂-dependent (SOD-inhibitable) reduction of ferricytochrome c, as previously described. Assays were performed in 12 × 75 mm polystyrene tubes. All experiments were performed in triplicate and were repeated at least twice for verification. A suspension of neutrophils (7.5 × 10⁶ cells) was incubated at 37 °C for ten minutes prior to the addition of LT₄ or its solvent. After an additional 37 °C incubation (two minutes unless otherwise noted), 1.5 × 10⁶ cells was pipetted into cytochrome c solutions (final concentration 0.1 mmol/L) and cell suspensions incubated with fMLP (10⁻⁵ mol/L), PMA (3.2 × 10⁻⁸ mol/L), or opsonized zymosan (250 μg/mL). The reaction mixtures were then centrifuged to remove cells, and the extent of cytochrome c reduction in each supernatant was determined to determine the influence of LTB₄ on the reaction of PMNs with LT₄. The reaction of PMNs with LT₄ was incubated at 37 °C for five minutes. After addition of fMLP, cells were incubated at 37 °C for five minutes, and the reaction subsequently stopped by placing the suspensions in a melting ice bath. Aliquots of 500 μL from each sample were layered onto 500 μL of 25% Apiezon C oil in dibutyl phthalate in 1.5-mL microfuge tubes (Eppendorf Microtest tube, Brinkman, Inc., Westbury, NY) and pelleted by a two-minute spin at 13,000 g in a table-top centrifuge (Micro-centrifuge 235B, Fisher Scientific, Pittsburgh, Pa). After centrifugation, the aqueous and oil layers were aspirated and the tip of each tube containing the cell pellet was cut off into a scintillation vial. In order to solubilize the pellet, 500 μL of 0.75N NaOH was added to each vial. Once the cells were dissolved, 10 mL of scintillation fluid, prepared as previously described, was added and vials were counted using a Beckman LS-250 liquid scintillation counter. Specific binding was determined by subtracting nonspecific binding from total binding.

Statistical Analysis

Tests for significance were performed using the two-tailed Student's t-test for paired or unpaired data, as appropriate.

RESULTS

Effect of LTB₄ on Neutrophil O₂ Release

Figure 1 demonstrates the effect of LTB₄ on PMN O₂ production evoked by exposure to PMA, zymosan, or fMLP. Preincubation of PMNs with LTB₄ in concentrations from 6.7 × 10⁻¹⁵ to 6.7 × 10⁻⁸ mol/L had no effect on O₂ release following stimulation with PMA or zymosan. In contrast, preincubated cells subsequently exposed to 10⁻⁷ mol/L fMLP demonstrated a consistent enhancement of O₂ production. Enhancement was maximal with an LTB₄ concentration of 1 to 10 nmol/L (180% ± 41% of control at 10 nmol/L, n = 23), but consistent enhancement was demonstrated at LTB₄ concentrations as low as 10⁻¹⁰ mol/L (140% ± 22% of control, n = 12). In the absence of fMLP, LTB₄ stimulated O₂ release above resting values only at concentrations greater than 10⁻⁷ mol/L (Table 1). Thus, the LTB₄ concentrations used in these experiments (10⁻¹² to 10⁻⁸ mol/L) were below those that yielded detectable O₂ production.

We have noted wide variation in the response of fMLP-stimulated control cells (preincubated with
BSA alone) obtained from different donors. In the present studies, control values for O2 production ranged from 4.17 to 24.52 nmol O2/1.5 x 106 neutrophils. Despite this variability, LTB4-treated cells demonstrated enhanced fMLP-induced O2 production. Mean O2 generation by control cells was 11.92 ± 5.22 nmol O2/1.5 x 106 neutrophils, compared with 20.20 ± 6.92 nmol O2 for cells exposed to 10-8 mol/L LTB4 (n = 23, P < .001).

In order to determine whether the enhancing effect of LTB4 required the continuing presence of the lipid during exposure to fMLP, neutrophils incubated with 10-8 mol/L LTB4 were centrifuged and washed twice to remove the lipid from the medium. After resuspen-

Fig 1. Effect of LTB4 on neutrophil O2 release. Aliquots of 1.5 x 106 PMNs were incubated with LTB4 for two minutes at 37°C prior to exposure to fMLP (10-7 mol/L) for five minutes. PMA (3.2 x 10-8 mol/L for ten minutes, or opsonized zymosan (OZ, 250 µg/mL) for 20 minutes. For each stimulus, the effect of LTB4 is presented as the percent of control response obtained with the addition of BSA, 50 mg/mL (solvent), in place of LTB4. Data points represent the means and error bars the standard deviations (SD) of triplicate determinations. Concentrations of LTB4 indicated are those in the final incubation medium. Preincubation concentrations were 6.7-fold higher.

Table 1. Effect of LTB4 as a Primary Oxidative Stimulus and as an Irreversible Enhancer of fMLP-Stimulated O2 Release

<table>
<thead>
<tr>
<th>Incubation Conditions</th>
<th>nmol O2/1.5 x 106 PMN</th>
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<tbody>
<tr>
<td>Buffer*</td>
<td>7.45 ± 0.63†</td>
</tr>
<tr>
<td>LTB4 2 x 10-6 mol/L</td>
<td>8.90 ± 0.81</td>
</tr>
<tr>
<td>LTB4 2 x 10-7 mol/L</td>
<td>8.11 ± 0.48</td>
</tr>
<tr>
<td>LTB4 2 x 10-8 mol/L</td>
<td>7.40 ± 0.54</td>
</tr>
<tr>
<td>Buffer + fMLP‡</td>
<td>11.27 ± 0.20</td>
</tr>
<tr>
<td>LTB4 10-6 mol/L (washed) + fMLP</td>
<td>17.74 ± 0.22 (157)§</td>
</tr>
</tbody>
</table>

*Neutrophils were incubated for five minutes at 37°C with either buffer (BSA) or the indicated concentration of LTB4. †Numbers represent mean ± standard deviation (SD) of triplicate determinations. ‡Neutrophils were incubated for five minutes with either buffer (BSA) or 10-6 mol/L LTB4, diluted in HBSS, and subsequently washed twice by centrifugation. Cells were then resuspended in HBSS with cytochrome c (0.1 mmol/L) and exposed to fMLP (10-7 mol/L) for five minutes at 37°C. §Percent control response.

Table 2. Effect of LTB4 Preincubation Time on Enhancement of fMLP-Stimulated O2 Production

<table>
<thead>
<tr>
<th>Preincubation Time (min)*</th>
<th>Percent Control O2 Response†</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>98 ± 5</td>
</tr>
<tr>
<td>1</td>
<td>103 ± 5</td>
</tr>
<tr>
<td>3</td>
<td>144 ± 5</td>
</tr>
<tr>
<td>5</td>
<td>174 ± 4</td>
</tr>
<tr>
<td>15</td>
<td>179 ± 2</td>
</tr>
<tr>
<td>30</td>
<td>194 ± 5</td>
</tr>
<tr>
<td>45</td>
<td>162 ± 1</td>
</tr>
<tr>
<td>60</td>
<td>135 ± 3</td>
</tr>
</tbody>
</table>

*Times represent duration of exposure of PMNs to 6.7 x 10-8 mol/L LTB4 prior to addition of 10-7 mol/L fMLP. †Mean ± SD of triplicate determinations of percent response of simultaneously run controls.

To ensure that LTB4 did not in some way interfere with the interaction between O2 and cytochrome c, we determined the effect of the lipid on a cell-free O2-generating system. As previously described, the oxidation of xanthine by xanthine oxidase in air produces O2 as measured by the SOD-inhibitable reduction of cytochrome c. LTB4 had no effect on the rate of cytochrome c reduction in this system compared with controls, wherein BSA was substituted for LTB4 (3.81 ± 0.32 nmol O2/min in controls v 3.74 ± 0.52 nmol O2/min for LTB4, n = 6, P > .1).

In order to determine whether the degree of enhancement by LTB4 was dependent on the time of exposure of neutrophils to the lipid, cells were preincubated with LTB4 for various times prior to stimulation with fMLP. As seen in Table 2, the simultaneous exposure of neutrophils to LTB4 and fMLP resulted in no enhancement. Preincubation with LTB4 for longer periods resulted in augmented O2 release, with maximum enhancement occurring with preincubations of five to 30 minutes. After 30 minutes of preincubation, the percentage enhancement decreased, although enhancement was seen after incubation periods up to 60 minutes. This diminution in the enhancement phenomenon could be due to metabolism or degradation of LTB4 in our system. However, with prolonged incubation at 37°C, neutrophils became more activated, and the lesser degrees of enhancement seen after such periods may reflect control values that are closer to maximal responses and, thus, cannot be enhanced to as great a degree as lower control values.

Table 3 demonstrates the effect of extracellular Ca++ and Mg++ on oxidative enhancement by LTB4. fMLP-mediated O2 release from control cells was decreased in the absence of Ca++ and Mg++ (55% of values obtained in the presence of the cations). Despite the absence of extracellular cations, PMNs exposed to
LTB
4 ENHANCEMENT OF PMN O2 RELEASE

LTB
4 generated increased amounts of O2 compared with controls. The degree of enhancement was the same (158% of respective controls) regardless of the presence or absence of Ca++ and Mg++. In addition, the absence of the cations resulted in the same degree of inhibition of O2 release (4% inhibition of O2 release in the presence of Ca++ and Mg+++) for both controls and LTB
4-treated cells.

Effect of LTB
4 on fMLP-Induced Chemiluminescence

The emission of light from stimulated neutrophils (chemiluminescence) is felt to result from one or more of several oxidizing species13-15 and is thus another measure of neutrophil oxidative metabolism. To further assess the effect of LTB
4 on chemotactic peptide-stimulated oxidative metabolism, neutrophils were preincubated with LTB
4 under the same conditions used in O2 assays. Figure 2 demonstrates the results of one representative experiment in which LTB
4-treated cells generated a markedly higher chemiluminescent response than controls (peak cpm in LTB
4-treated neutrophils - 323% of control). In the absence of fMLP, neutrophils exposed to LTB
4 exhibited a brief burst of chemiluminescence, but only at concentrations greater than 10 ^7 mol/L (data not shown).

Effect of LTB
4 on fMLP Receptor Binding

Because other degranulating stimuli have been shown to increase the number of fMLP receptors on the PMN plasma membrane,16 we investigated the effect of LTB
4 on binding of fMLP in order to assess an LTB
4-induced increase in fMLP receptors as a possible mechanism of oxidative enhancement. In our assay system, preincubation of PMNs with ionophore A23187 (2.5 x 10 ^8 mol/L) resulted in an approximate doubling of subsequent binding of 3H-fMLP to the cells (data not shown), in accordance with previous observations.16 For experiments with LTB
4, incubation conditions and times were chosen to most closely approximate those used in previous studies of O2 generation and chemiluminescence. Table 4 demonstrates the results of binding assays utilizing neutrophils obtained from a single donor. Specific binding of 3H-fMLP to neutrophils preincubated with 6.7 x 10 ^8 mol/L LTB
4 was 91.8% ± 2.4% of binding to control cells. Although the magnitude of fMLP binding varied among the individuals tested, this slight decrease in fMLP receptor binding in the presence of LTB
4 was seen when cells from two other donors were used under the same experimental conditions. When experiments using neutrophils from three different individuals are combined, binding of 3H-fMLP by LTB
4-treated cells was 89.8% ± 4.2% of controls (n = 8, P < .01). The slight decrease in fMLP receptor binding was not due to a decrease in cell viability, as the ability of cells to exclude trypan blue was similar in control and LTB
4-treated cells. Enhancement of fMLP receptor expression would not appear to be the mechanism whereby LTB
4 enhances oxidative responses to the peptide.

DISCUSSION

The role of leukotrienes and other products of arachidonic acid lipoxygenation in the inflammatory

Table 3. Effect of Cations on LTB
4 Enhancement of fMLP-Induced O2 Release

<table>
<thead>
<tr>
<th>No Cations</th>
<th>Ca++, Mg++</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMN + BSA + HBSS</td>
<td>2.97 ± 0.36†</td>
</tr>
<tr>
<td>PMN + BSA + fMLP</td>
<td>3.76 ± 0.65</td>
</tr>
</tbody>
</table>
| PMN + LTB
4 + fMLP | 5.94 ± 0.42 (158) | 10.88 ± 0.55 (158) |

*Numbers represent mean ± SD of triplicate determinations in a representative experiment using cells from a single donor. Qualitatively similar results were obtained using cells from several different donors.

†Percent of response obtained in the absence of LTB
4.

Table 4. Effect of LTB
4 on Neutrophil Binding of 3H-fMLP

<table>
<thead>
<tr>
<th>Counts per Minute (Specific Binding)</th>
<th>Percent Control Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control PMN</td>
<td>7,103.2 ± 147.3*</td>
</tr>
</tbody>
</table>
| LTB
4-treated PMN | 6,518.2 ± 167.9 | 91.76 ± 2.36 |

*Numbers represent cpm per 5 x 10^6 PMNs after incubation with 5 x 10 ^8 mol/L 3H-fMLP minus nonspecific binding (obtained by adding 1,000-fold excess of unlabeled fMLP) and are means ± SD of triplicate determinations from a single donor. Results from the same experiment using cells from two additional donors also revealed a slight decrease in 3H-fMLP bound by LTB
4-treated neutrophils (mean percent of control: 89.8% ± 4.2% for all three donors collectively, n = 8, P < .01).
process has only recently been recognized. Although leukotrienes C₄ and D₄ (the predominant components of SRS-A) have more potent effects on vascular, bronchial, and gut smooth muscle,¹⁷ LTB₄ has the most pronounced direct effects on neutrophil function. The chemotactic and chemokinetic properties of LTB₄ are well known. This leukotriene is an effective chemoattractant at nanomolar concentrations, making it equipotent with the chemotactic peptides fMLP and C₅a.²⁶ LTB₄ induces neutrophils to aggregate and degranulate, although, as with other chemoattractants, demonstration of these effects may require relatively high (micromolar) concentrations of the lipid or an in vitro factor, is unaffected.²⁶ Studies of aggregation and neutrophil responses to nonchemotactic stimuli, as shown by English et al.⁵ Enhancement of O₂ release is specific for that induced by fMLP, as O₂ production following exposure to PMA or zymosan was unaffected by LTB₄.

LTB₄ has recently been shown to modulate other neutrophil responses. O’Flaherty et al.¹⁹ have demonstrated that cells exposed to LTB₄ are inhibited or “desensitized to” aggregation induced by subsequent exposure to a variety of aggregating stimuli, including fMLP and LTB₄ itself. The same group of investigators have also noted that, whereas LTB₄ desensitizes neutrophils to degranulation evoked by subsequent exposure to LTB₄, the response to other aggregating stimuli, such as fMLP, C₅a, and platelet activating factor, is unaffected.²⁰ Studies of aggregation and degranulation emphasize the functional specificity of LTB₄ modulation of fMLP-stimulated neutrophil responses, as only the oxidative responses to the peptide are enhanced.

The mechanism whereby LTB₄ increases oxidative responsiveness to fMLP remains unclear. Fletcher et al.¹⁹ have demonstrated that some substances that cause neutrophil degranulation, including ionophore A23187 and PMA, increase both the number of fMLP receptors on the neutrophil plasma membrane and the cell’s oxidative response to fMLP. These authors suggest that neutrophil-specific granules provide a source of preformed fMLP receptors that can be translocated to the cell’s external surface during degranulation. Subsequent fMLP exposure would cause an increase in receptor–ligand coupling, with resultant enhancement of fMLP-mediated responses. Although LTB₄ does cause degranulation and specific granule enzyme release, a different mechanism of enhancement of fMLP-induced O₂ release appears to be operative. We were unable to demonstrate an increase in fMLP receptor binding using conditions under which LTB₄ enhances fMLP-induced O₂ release. We also noted enhancement at LTB₄ concentrations below those required to produce degranulation.⁵ LTB₄ could enhance the interaction between receptor–ligand binding and the process that leads to activation of the O₂-generating enzyme. LTB₄ causes an increase in neutrophil calcium uptake,¹ and as extracellular calcium is required for optimal PMN oxidative responses to fMLP,²¹ the enhancing effect of LTB₄ could be mediated through this divalent cation. Our demonstration that extracellular calcium is not required for LTB₄ to enhance fMLP-induced O₂ release strongly argues against LTB₄-mediated increases in calcium uptake as the mechanism of enhancement. From the present data, we cannot rule out an LTB₄ effect on intracellular calcium metabolism which might augment cellular responses to the chemotactic peptide.

We have recently found that other arachidonic acid lipoxygenase products also augment fMLP-induced O₂ production in neutrophils (unpublished observations). However, all of the compounds tested thus far are far less potent than LTB₄ in producing the enhancement, with most compounds requiring 100-fold greater concentrations. Investigation of a large number of lipoxygenase products is continuing in our laboratory.

In vivo, LTB₄ is produced by stimulated neutrophils and mononuclear phagocytes.³² This lipid, as well as other arachidonic acid lipoxygenation products, are found in inflammatory exudates, such as synovial fluid from chronically inflamed joints.²³ As neutrophils bathed in such fluid would be exposed to LTB₄, amplification of chemotactic factor-induced O₂ release could occur in vivo, with potential for increased oxygen radical-mediated tissue destruction. There also appears to be a physiologic role for endogenously produced LTB₄ in neutrophil function, which is in part suggested by studies demonstrating inhibition of neutrophil function by inhibitors of leukotriene synthesis (ETYA, nordihydroguaiaretic acid).³,²⁴ ²⁵ It is possible that LTB₄ and other arachidonate lipoxygenation products have effects on the cell of origin as well as effects on other cells after release into the extracellular medium. Delineation of the relative contributions of endogenous and exogenous LTB₄ to neutrophil function will require further study.
REFERENCES


Enhancement of chemotactic factor-stimulated neutrophil oxidative metabolism by leukotriene B4

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