Effect of Leukotriene B$_4$ on Enhancement of Superoxide Production Evoked by Formyl-Methionyl-Leucyl-Phenylalanine in Myeloid Differentiated HL-60 Cells: Possible Involvement of Intracellular Calcium Influx and High Affinity Receptor for Leukotriene B$_4$

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ABSTRACT
Exposure of a human leukemic cell line HL-60 to 1% dimethylsulfoxide (DMSO) for 4 days induced myeloid differentiation. DMSO-differentiated HL-60 cells displayed high and low-affinity binding sites for leukotriene B$_4$ (LTB$_4$). The pretreatment of myeloid differentiated HL-60 cells with 1-10 nM LTB$_4$ enhanced superoxide production evoked by 100 nM formyl-methionyl-leucyl-phenylalanine (fMLP) to 127-137% of the controls stimulated by fMLP alone. A concentration eliciting a half maximal increase (EC$_{50}$) of LTB$_4$ for the enhancing effect on superoxide production evoked by fMLP was 0.32 nM. This was roughly similar to the dissociation constant (Kd) of high affinity receptors for LTB$_4$ (0.23 nM). These results suggest that high affinity receptors transduce the enhancing effect of LTB$_4$ on fMLP-induced superoxide production. Although it seems possible that enhancement of fMLP-induced superoxide production is associated with a substantial increase and/or an affinity alteration in receptors for fMLP, LTB$_4$-pretreated cells failed to show significant changes in fMLP binding compared to non-pretreated ones. It seems likely that Ca$^{2+}$ influx transduces enhancement of fMLP-induced superoxide production, because extracellular Ca$^{2+}$ is necessary for an enhancing effect of fMLP-induced superoxide production. Also, EC$_{50}$ of LTB$_4$ for Ca$^{2+}$ influx (0.78 nM) was similar to that of the enhancing effect of superoxide generation evoked by fMLP. Although pretreatment of LTB$_4$ failed to enhance the maximal level of fMLP-induced intracellular Ca$^{2+}$ rise, transient overshoot in intracellular Ca$^{2+}$ evoked by fMLP declined more rapidly after LTB$_4$ pretreatment. Possible involvement of high affinity binding sites for LTB$_4$ and Ca$^{2+}$ influx was noted in the LTB$_4$-enhancement of fMLP-induced superoxide production in DMSO-differentiated HL-60 cells. However, the significance of the rapid attenuation of intracellular Ca$^{2+}$ overshoot needs further evaluation.

Key words: Leukotriene B$_4$, HL-60 cells, Superoxide, Calcium

The HL-60 cell line of a patient with leukemia, initially diagnosed as acute promyelocytic leukemia$^9$ but now classified as acute myeloblastic leukemia with maturation$^5$, undergoes differentiation to a number of different cell types by a variety of different compounds. DMSO-induced myeloid differentiated HL-60 cells exhibit functional maturity$^{5,17}$, possessing the binding sites for the chemotactic peptide fMLP$^{18}$ and LTB$_4$,$^3$ as well as the ability to produce LTB$_4$$^{29}$. DMSO-differentiated HL-60 cells provide a convenient system for assessing the synergistic effect of neutrophil stimuli on granulocyte functions. It should be kept in mind, however, that the data were obtained from a heterogeneous cell population. Experiments with HL-60 cells have many advantages in studies of receptor processing and functioning, because of these cells' long life span in vitro.

LTB$_4$ is a 5-lipoxygenase metabolite of arachidonic acid which is produced by neutrophils in response to specific stimuli$^{12,24}$, and shares many proinflammatory properties, including the ability to stimulate neutrophil adherence and chemotaxis$^{20,24,25}$. Although LTB$_4$ is a poor stimulant of neutrophil superoxide, it has been known to enhance fMLP-induced respiratory burst$^{10,11}$. However, in myeloid differentiated HL-60 cells, the enhancing effect of LTB$_4$ on superoxide production has not yet been established. Experimental evidence has shown that an alternation in expression of receptors$^{8,27}$ and/or Ca$^{2+}$ mobilization$^7$ modifies the cellular response of neutrophils to fMLP. It seems likely that LTB$_4$-enhancement of superoxide production evoked by fMLP is mediated by alteration in expression of receptors and/or Ca$^{2+}$ influx.
This report shows that LTB₄ increased fMLP-induced superoxide production in myeloid differentiated HL-60 cells. The mechanisms of this enhancing effect of LTB₄ in DMSO-induced mature HL-60 cells were examined. This study has demonstrated that high-affinity receptors for LTB₄ transduce not only Ca²⁺ influx but also have an increasing effect on fMLP-induced superoxide generation. It is shown also that transient overshoot in intracellular Ca²⁺ in LTB₄-treated cells declines more rapidly compared to that of LTB₄ non-pretreated cells.

MATERIALS AND METHODS

Reagents

LTB₄ (Paesel, Frankfurt, FRG), fMLP (Protein Research Laboratory, Osaka, Japan), DMSO (Merk, Darmstock, FRG), ferricytochrome c Type VI, (Sigma, St.Louis, MO), Ethylene glycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA, Nakarai, Kyoto, Japan), crystal ovalbumin (OVA, Sigma), Fura 2/AM (Dojindo, Kumamoto, Japan), Hanks' balanced salt solution (HBSS, Gibco, Grand Island, NY), NCS tissue solubilizer (Amersham, Arlington Heights, IL), [³H] LTB₄ (32 Ci/mmol, New England Nuclear, Boston, MA) and [³H] fMLP (60 Ci/mmol, New England Nuclear) were obtained from the suppliers noted. [³H] LTB₄ and [³H] fMLP were stored in ethanol at -20°C and evaporated by centrifugal evaporator (Yamato, Tokyo, Japan). Where low extracellular Ca²⁺ was specified for incubation, Ca²⁺-free HBSS containing 1 mM EGTA was used.

Fura 2 loading and measurement of intracellular Ca²⁺

The intracellular levels of Ca²⁺ were inferred from measurement of fluorescence of Ca²⁺ indicator Fura 2 as previously described[16,23]. To achieve uptake of Fura 2, 10⁶ cells were incubated with 2 µM Fura 2/AM for 30 min at 37°C in 1 ml of HBSS. The cells were diluted twofold and incubated for an additional 30 min at room temperature. The Fura 2-loaded cells were washed several times and resuspended in medium containing 130 mM NaCl, 5 mM KCl, 5.5 mM glucose, 1 mM MgCl₂, 1.5 mM CaCl₂ and 20 mM Hepes buffer (pH 7.2) at a concentration of 5 x 10⁶ cells/ml. The fluorescence of Fura 2-loaded cells was recorded with a spectrophurometer (Hitachi model MPF-4, Hitachi, Tokyo, Japan) at an excitation wavelength of 335 nm and an emission wavelength of 360 nm. Intracellular Ca²⁺ levels were calculated as previously described[20].

Assay for LTB₄ receptors

Specific binding of [³H] LTB₄ to HL-60 cells was measured as previously described[16]. Briefly, 10⁷ cells/ml were incubated on ice for 60 min in the presence of 0.1 to 40 nM [³H] LTB₄ in a final volume of 200 µl of Ca²⁺-free HBSS-OVA (HBSS containing 0.1 g OVA per 100 ml) containing 1 mM EGTA. The reaction was terminated by a rapid filtration through Whatman GF/C glass fiber filter (Whatman, Maidstone, England) and the filters were immediately washed with 10 ml ice cold HBSS. The filters were air dried and solubilized in 1 ml of NCS solution at 50°C for 60 min in a scintillation vial and the radioactivity was determined by liquid scintillation spectrometry. Nonspecific binding was defined as the amount of [³H] LTB₄ bound in the presence of a 500-fold excess of unlabeled LTB₄. The binding data were analyzed by the method of Rosenthal[22] as previously reported[19] on a NEC PC-9801 microcomputer system (NEC, Tokyo, Japan).

[³H] fMLP binding to LTB₄-treated HL-60 cells

Forty million per milliliter of HL-60 cells in HBSS were incubated with or without 100 nM LTB₄ for 5 min at 37°C, and then equal volume of Ca²⁺-free HBSS containing 2 mM EGTA was added. Specific binding of [³H] fMLP of HL-60 cells was measured as previously described, with some modifications[8]. Briefly, 2 x 10⁶ cells were incubated with different concentrations of [³H] fMLP (0.1—400 nM) in a total volume of 200 µl of Ca²⁺-free HBSS-OVA in the presence or absence of 500-fold excess of unlabeled fMLP. After incubation for 60 min, the cell suspensions were rapidly filtered onto Whatman GF/C glass fiber filters, which were washed with ice-cold HBSS. The radi-
oactivity was counted as described in LTB₄ binding assay.

Statistical analysis
Statistical analysis was performed by the two-tailed Student’s t-test and paired t-test.

RESULTS
Effect of 1% DMSO on myeloid differentiation of HL-60 cells

When the subclone of HL-60 cells was cultured for 4 days in the presence of 1% DMSO, the cells differentiated into promyelocytes (5.8 ± 10.3%), myelocytes (29 ± 3.3%), metamyelocytes (23.6 ± 7.4%), band cells (15.2 ± 5.4%) and segmented cells (26.2 ± 5.6%). Although myeloid differentiated HL-60 cells produced superoxide by a stimulation of fMLP, immature HL-60 cells failed to produce superoxide even by stimulation with fMLP following LTB₄ (data not shown). Myeloid differentiated HL-60 cells had two binding sites for LTB₄. Kd for high affinity receptor (KdH) was 0.23 nM and Kd for low affinity receptors (KdL) was 30.3 nM (Table 1).

Effect of LTB₄ on fMLP-induced superoxide production

Preincubation of myeloid differentiated HL-60 cells with LTB₄ for 5 min, in a dose dependent fashion, enhanced superoxide production evoked by 100 nM fMLP (Table 2). One hundred nM LTB₄ increased superoxide production by 137% at 100 nM fMLP. EC₅₀ of LTB₄ for enhancement of fMLP-induced superoxide production was 0.32 nM.

Table 1. LTB₄ Receptors on Myeloid Differentiated HL-60 Cells

<table>
<thead>
<tr>
<th>Affinity</th>
<th>Kd nM</th>
<th>Bmax fmol/10⁷ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>0.23 ± 0.10</td>
<td>30.3 ± 0.78</td>
</tr>
<tr>
<td>Low</td>
<td>12.4 ± 3.38</td>
<td>335.7 ± 72.8</td>
</tr>
</tbody>
</table>

Myeloid differentiated HL-60 cells were incubated with 0.1 to 40 nM [³H] LTB₄ with or without 500-fold unlabeled LTB₄ in Ca²⁺ free HBSS-OVA for 60 min on ice (n=3).

Table 2. Effect of LTB₄ on Superoxide Production Evoked by fMLP in Myeloid Differentiated HL-60 Cells

<table>
<thead>
<tr>
<th>Concentration of LTB₄ (nM)</th>
<th>Superoxide production nmol/min/10⁶ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.00 ± 0.25</td>
</tr>
<tr>
<td>0.1</td>
<td>3.77 ± 0.63</td>
</tr>
<tr>
<td>1</td>
<td>5.08 ± 0.22*</td>
</tr>
<tr>
<td>10</td>
<td>5.42 ± 0.30*</td>
</tr>
<tr>
<td>100</td>
<td>5.48 ± 0.30*</td>
</tr>
</tbody>
</table>

Myeloid differentiated HL-60 cells were incubated for 5 min at 37°C with different concentrations of LTB₄ before addition of 100 nM fMLP (n=4).

*p<0.01 as compared with the control.

This was roughly similar to KdH for LTB₄ (0.23 nM).

Effect of LTB₄ on [³H] fMLP binding

Differentiated HL-60 cells expressed both high and low-affinity receptors for fMLP. Scatchard analysis of specific binding of [³H] fMLP in LTB₄-pretreated myeloid differentiated HL-60 cells expressed as KdH of 1.83 ± 1.26 nM and KdL of 36.07 ± 24.6 nM (n=3). Total binding capacity (Bmax) for high affinity receptors (BmaxH) was 39.1 ± 8.8 fmol/10⁷ cells, and Bmax for low affinity receptors (BmaxL) was 436.8 ± 126.1 fmol/10⁷ cells in LTB₄-pretreated cells. In non-pretreated cells KdH was 0.90 ± 0.32 nM, KdL 73.9 ± 4.6 nM, BmaxH 48.3 ± 24.0 fmol/10⁷ cells, and BmaxL 416.7 ± 115.5 fmol/10⁷ cells in non-pretreated cells (Fig. 1). The difference in fMLP binding affinity and the number of binding sites exhibited by the LTB₄-pretreated cells compared with non-pretreated cells was not statistically significant (p>0.05).

Role of Ca²⁺ in modulation of superoxide production

When the cells were suspended in Ca²⁺-free HBSS containing 1 mM EGTA, fMLP-induced superoxide production of LTB₄-pretreated myeloid differentiated HL-60 cells and non-pretreated cells was 1.15 ± 0.10 and 1.36 ± 0.21 nmol O₂⁻/min/10⁶ cells (p>0.05, n=3, Fig.2). In Ca²⁺ containing HBSS, fMLP-induced superoxide production of LTB₄-pretreated cells and non-pretreated cells were 6.07 ± 0.31 and 3.97 ± 0.21 nmol O₂⁻/min/10⁶ cells (p<0.01, n=3). Although pretreatment with LTB₄ failed to enhance fMLP-induced superoxide generation in the absence of Ca²⁺, the stimulation of Ca²⁺-containing HBSS with or without 100 nM LTB₄ for 5 min at 37°C followed by [³H] fMLP binding assay on ice.
Fig. 2. Effect of elimination of extracellular Ca\textsuperscript{2+} on LTB\textsubscript{4}-induced enhancement of superoxide production evoked by fMLP. Myeloid differentiated HL-60 cells were preincubated with 100 nM LTB\textsubscript{4} or HBSS for 5 min before addition of 100 nM fMLP. Cells were incubated in HBSS containing Ca\textsuperscript{2+} (upper figures) or in Ca\textsuperscript{2+}-free HBSS containing 1 mM EGTA (lower figures).

Fig. 3. LTB\textsubscript{4}-induced intracellular Ca\textsuperscript{2+} mobilization of myeloid differentiated HL-60 cells. Maximal level of intracellular Ca\textsuperscript{2+} mobilization of the cells stimulated with 0.1, 1, 10 and 100 nM of LTB\textsubscript{4} was 123 ± 25.3 nM, 194.4 ± 7.8 nM, 245.2 ± 16.5 nM and 248.2 ± 19.7 nM, respectively. Base-line intracellular Ca\textsuperscript{2+} was 113 ± 8.5 nM. Triplicate determinations were performed in each condition.

Fig. 4. Effect of LTB\textsubscript{4} on fMLP-induced intracellular Ca\textsuperscript{2+} mobilization in myeloid differentiated HL-60 cells. The decline of the transient overshoot is presented as tangent D, where D is the angle between the base line and the tangential line of the decreasing phase of transient intracellular Ca\textsuperscript{2+} mobilization that draws from the peak point to a level at 30 sec later. Tangent D of the decreasing phase of intracellular Ca\textsuperscript{2+} in the cells pretreated with PBS or 100 nM LTB\textsubscript{4} was 1.22 ± 0.23 and 2.00 ± 0.23*, respectively. Triplicate determinations were performed in each condition.

* p < 0.05 as compared to PBS pretreated cells.

Extracellular Ca\textsuperscript{2+}, LTB\textsubscript{4} significantly enhanced fMLP-induced superoxide production in Ca\textsuperscript{2+} containing HBSS. LTB\textsubscript{4} itself induced intracellular Ca\textsuperscript{2+} rise in a dose dependent manner (Fig. 3). EC\textsubscript{50} for LTB\textsubscript{4} to increase intracellular Ca\textsuperscript{2+} level in myeloid differentiated HL-60 cells was 0.78 nM, which was also approximately similar to \( K_{\text{diss}} \) for LTB\textsubscript{4}.

However, fMLP-induced intracellular Ca\textsuperscript{2+} rise was not affected by LTB\textsubscript{4} pretreatment (Fig. 4). The maximal level of increase of 100 nM fMLP-induced intracellular Ca\textsuperscript{2+} in 100 nM LTB\textsubscript{4}-pretreated cells (332.6 ± 49.3 nM) was similar to that of non-pretreated ones (330.7 ± 65.9 nM). When LTB\textsubscript{4} (100 nM)-pretreated cells were stimulated with 1 nM and 10 nM fMLP, the maximal level of intracellular Ca\textsuperscript{2+} was also similar to that of the non-pretreated ones (data not shown). The transient overshoot in intracellular Ca\textsuperscript{2+} induced by fMLP in LTB\textsubscript{4}-pretreated cells declined more rapidly compared to that of the cells stimulated with fMLP alone. Similar rapid declines of in-
tracellular Ca\(^{2+}\) were observed when differentiated HL-60 cells were pretreated with 1 nM and 10 nM LTB\(_4\) before stimulation with 100 nM fMLP (data not shown).

**DISCUSSION**

It is a widely accepted hypothesis that cell surface receptors regulate the responses of the cells. In this study, pretreatment of the myeloid differentiated HL-60 cells with 1–100 nM LTB\(_4\) increased fMLP-evoked superoxide production to 127–137% of the control. EC\(_{50}\) of LTB\(_4\) for an enhancing effect of superoxide production evoked by fMLP was roughly similar to \(K_{dH}\) for LTB\(_4\). These results suggest that high-affinity receptors for LTB\(_4\) transduce an enhancing effect of superoxide production evoked by fMLP.

It is well known that several substances, including Ca\(^{2+}\) ionophore A23187 and PMA, increase both the number of fMLP receptors on the neutrophil plasma membrane and the cellular oxidative response to fMLP\(^{10,18,37-39}\). It seems possible that subsequent fMLP exposure can cause an increase and/or an affinity change in receptor-ligand coupling, resulting in an enhancement of fMLP-mediated responses. However, in the present data, pretreatment of LTB\(_4\) failed to cause significant changes in high and low-affinity receptors for fMLP in myeloid differentiated HL-60 cells. In human neutrophils, LTB\(_4\) also failed to alter fMLP binding in spite of enhancing fMLP-induced NBT reduction\(^{10}\). It seems likely that an enhancing effect of LTB\(_4\) is not mediated by a change in the number and affinity of fMLP binding sites. However, because the binding studies were performed in heterogeneous differentiated HL-60 cells, modulation of receptor number and/or affinity of a subpopulation of cells cannot be ruled out.

Since LTB\(_4\) is a calcium ionophore\(^{39}\), the role of calcium in the enhancing effect was also investigated. fMLP-induced superoxide production was enhanced by pretreatment of LTB\(_4\) in the presence of extracellular Ca\(^{2+}\). However, in the absence of Ca\(^{2+}\), an enhancing effect of LTB\(_4\) was not observed. These results suggest that the extracellular Ca\(^{2+}\) is necessary for an enhancing effect of LTB\(_4\) on superoxide production evoked by fMLP in myeloid differentiated HL-60 cells. EC\(_{50}\) of LTB\(_4\) for intracellular calcium mobilization was approximately similar to that of LTB\(_4\) for the enhancing effect on fMLP-induced superoxide production. Therefore, it seems likely that Ca\(^{2+}\) influx evoked by LTB\(_4\) transduces enhancement of fMLP-induced superoxide production.

Pretreatment of LTB\(_4\) did not alter the maximal level of increase of fMLP-induced intracellular Ca\(^{2+}\). The maximal intracellular Ca\(^{2+}\) level reflects mainly Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores\(^{29}\). Therefore, the enhancing effect of LTB\(_4\) may not be associated with changes in fMLP-induced Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores.

It is interesting that the transient overshoot in intracellular Ca\(^{2+}\) in LTB\(_4\)-treated cells declined more rapidly compared to LTB\(_4\), non-pretreated cells. The possibility that plasma membrane ionic channels are modified by a protein kinase C-dependent mechanism has been reported\(^{29}\). In myeloid differentiated HL-60 cells, inositol trisphosphate is thought to be a signal for fMLP-induced Ca\(^{2+}\) mobilization\(^{39}\). It seems likely that a rapid decrease in intracellular Ca\(^{2+}\) transient may indicate a change of activation of protein kinase C and/or phospholipase C in LTB\(_4\)-treated cells. The significance of LTB\(_4\) induced rapid decline of Ca\(^{2+}\) influx must be further evaluated.

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