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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[4] but now classified as acute myeloblastic leukemia with maturation[6], 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[1], 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[3,12,24], 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[8,9,27] 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 LTB_4 increased fMLP-induced superoxide production in myeloid differentiated HL-60 cells. The mechanisms of this enhancing effect of LTB_4 in DMSO-induced mature HL-60 cells were examined. This study has demonstrated that high-affinity receptors for LTB_4 transduce not only Ca^{2+} influx but also have an increasing effect on fMLP-induced superoxide generation. It is shown also that transient overshoot in intracellular Ca^{2+} in LTB_4 treated cells declines more rapidly compared to that of LTB_4 non-pretreated cells.

MATERIALS AND METHODS

Reagents

LTB_4 (Paesel, Frankfurt, FRG), fMLP (Protein Research Laboratory, Osaka, Japan), DMSO (Merk, Darmstock, FRG), ferricytochrome c TypeVI, (Sigma, St.Louis, MO), Ethylene glycol-bis-(ß-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), [^{3}H] LTB_4 (32 Ci/mmol, New England Nuclear, Boston, MA) and [^{3}H] fMLP (60 Ci/mmol, New England Nuclear) were obtained from the suppliers noted. [^{3}H] LTB_4 and [^{3}H] fMLP were stored in ethanol at -20°C and evaporated by centrifugal evaporator (Yamato Scientific model RD-41, Tokyo, Japan) and dissolved in Ca^{2+}-free HBSS before use.

Cells

HL-60 cells obtained from the Japan Cancer Research Resources Bank (Tokyo, Japan) were cloned by limiting dilution method and maintained in RPMI 1640 (GIBCO) supplemented with 10% fetal bovine serum (FBS, KC Biological, Lenexa, KS), 2 mM l-glutamine and 1% penicillin-streptomycin (GIBCO) as previously described[4]. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. For the experiments, HL-60 cells were cultured as previously described[5], but in 5% FBS and at 2—4 × 10^5 cells/ml in the presence of 1% DMSO for 4 days. For morphological assessment of the cells, cytospin slides were prepared using a Shandon Elliot cytospin centrifuge (Shandon Southern Products, Runcorn, England) and stained by Wright's method. The viability of the cells, assessed by trypan blue dye exclusion, was greater than 85%.

Assay for superoxide production

Superoxide generation was measured spectrophotometrically at 37°C by incubating 5 × 10^5 cells in a total volume of 1 ml HBSS in the presence of 100 μM of ferricytochrome c[16]. After 5 min preincubation with or without different concentrations of LTB_4 (0.1—100 nM), 100 nM fMLP was added. Cytochrome c reduction was recorded at 550—540 nm on a double wave length spectrophotometer (Hitachi model 557, Hitachi, Tokyo, Japan). Where low extracellular Ca^{2+} was specified for incubation, Ca^{2+}-free HBSS containing 1 mM EGTA was used.

Fura 2 loading and measurement of intracellular Ca^{2+}

The intracellular levels of Ca^{2+} were inferred from measurement of fluorescence of Ca^{2+} indicator Fura 2 as previously described[15,23]. To achieve uptake of Fura 2, 10^7 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 $MgCl_2$, 1.5 mM $CaCl_2$ and 20 mM Hepes buffer (pH 7.2) at a concentration of 5 × 10^6 cells/ml. The fluorescence of Fura 2-loaded cells was recorded with a spectroflurometer (Hitachi model MPF-4, Hitachi, Tokyo, Japan) at an excitation wavelength of 335 nm and an emission wavelength of 500 nm. Intracellular Ca^{2+} levels were calculated as previously described[15].

Assay for LTB_4 receptors

Specific binding of [^{3}H] LTB_4 to HL-60 cells was measured as previously described[14]. Briefly, 10^7 cells/ml were incubated on ice for 60 min in the presence of 0.1 to 40 nM [^{3}H] LTB_4 in a final volume of 200 μl of Ca^{2+}-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 number of [^{3}H] LTB_4 bound in the presence of a 500-fold excess of unlabeled LTB_4. 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).

[^{3}H] fMLP binding to LTB_4-treated HL-60 cells

Forty million per milliliter of HL-60 cells in HBSS were incubated with or without 100 nM LTB_4 for 5 min at 37°C, and then equal volume of Ca^{2+}-free HBSS containing 2 mM EGTA was added. Specific binding of [^{3}H] fMLP of HL-60 cells was measured as previously described, with some modifications[8]. Briefly, 2 × 10^6 cells were incubated with different concentrations of [^{3}H] fMLP (0.1—400 nM) in a total volume of 200 μl of Ca^{2+}-free HBSS-OVA in the presence or absence of 500-fold excess of unlabeled fMLP. After incubation for 60 min, the cell suspesions 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 LTB_4 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%, mean ± SD, n=5), 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 LTB_4 (data not shown). Myeloid differentiated HL-60 cells had two binding sites for LTB_4. Kd for high affinity receptor (K_{dH}) was 0.23 nM and Kd for low affinity receptor (K_{dL}) was 30.3 nM (Table 1).

Effect of LTB_4 on fMLP-induced superoxide production

Preincubation of myeloid differentiated HL-60 cells with LTB_4 for 5 min, in a dose dependent fashion, enhanced superoxide production evoked by 100 nM fMLP (Table 2). One hundred nM LTB_4 increased superoxide production by 137% at 100 nM fMLP. EC_{50} of LTB_4 for enhancement of fMLP-induced superoxide production was 0.32 nM.

Table 1. LTB_4 Receptors on Myeloid Differentiated HL-60 Cells

Affinity	Kd nM	B_{max} fmol/10^7 cells
High	0.23 ± 0.10	30.3 ± 0.78
Low	12.4 ± 3.38	335.7 ± 72.8

Myeloid differentiated HL-60 cells were incubated with 0.1 to 40 nM [^{3}H] LTB_4 with or without 500-fold unlabeled LTB_4 in Ca^{2+} free HBSS-OVA for 60 min on ice (n=3).

Table 2. Effect of LTB_4 on Superoxide Production Evoked by fMLP in Myeloid Differentiated HL-60 Cells

Concentration of LTB_4 (nM)	Superoxide production nmol/min/10^6 cells
0	4.00 ± 0.25
0.1	3.77 ± 0.63
1	5.08 ± 0.22*
10	5.42 ± 0.30*
100	5.48 ± 0.30*

Myeloid differentiated HL-60 cells were incubated for 5 min at 37°C with different concentrations of LTB_4 before addition of 100 nM fMLP (n=4).
*$p<0.01$ as compared with the control.

This was roughly similar to K_{dH} for LTB_4 (0.23 nM).

Effect of LTB_4 on [^{3}H] fMLP binding

Differentiated HL-60 cells expressed both high and low-affinity receptors for fMLP. Scatchard analysis of specific binding of [^{3}H] fMLP in LTB_4-pretreated myeloid differentiated HL-60 cells expressed as K_{dH} of 1.83 ± 1.26 nM and K_{dL} of 36.07 ± 24.6 nM (n=3). Total binding capacity (B_{max}) for high affinity receptors (B_{maxH}) was 39.1 ± 8.8 fmol/10^7 cells, and B_{max} for low affinity receptors (B_{maxL}) was 436.8 ± 126.1 fmol/10^7 cells in LTB_4-pretreated cells. In non-pretreated cells K_{dH} was 0.90 ± 0.32 nM, K_{dL} 73.9 ± 4.6 nM, B_{maxH} 48.3 ± 24.0 fmol/10^7 cells, and B_{maxL} 416.7 ± 115.5 fmol/10^7 cells in non-pretreated cells (Fig. 1). The difference in fMLP binding affinity and the number of binding sites exhibited by the LTB_4-pretreated cells compared with non-pretreated cells was not statistically significant ($p>0.05$).

Role of Ca^{2+} in modulation of superoxide production

When the cells were suspended in Ca^{2+}-free HBSS containing 1 mM EGTA, fMLP-induced superoxide production of LTB_4-pretreated myeloid differentiated HL-60 cells and non-pretreated cells was 1.15 ± 0.10 and 1.36 ± 0.21 nmol O_2^-/min/10^6 cells ($p>0.05$, n=3, Fig.2). In Ca^{2+} containing HBSS, fMLP-induced superoxide production of LTB_4 pretreated cells and non-pretreated cells were 6.07 ± 0.31 and 3.97 ± 0.21 nmol O_2^-/min/10^6 cells ($p<0.01$, n=3). Although pretreatment with LTB_4 failed to enhance fMLP-induced superoxide generation in the absence of

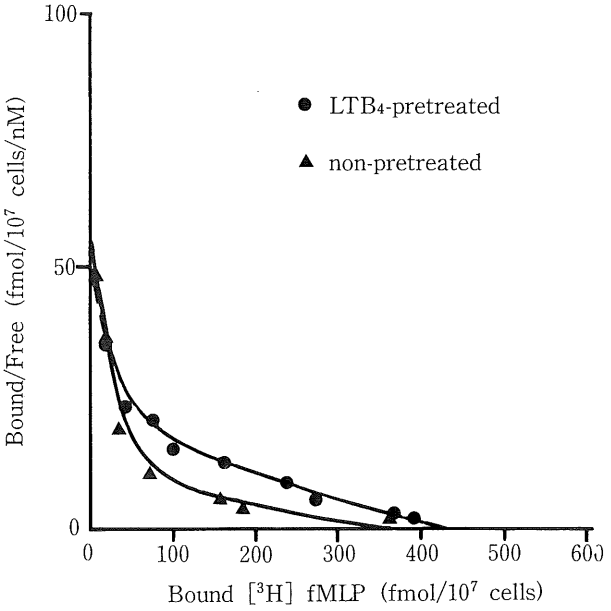

Fig. 1. Effect of LTB_4 on [^{3}H] fMLP binding to myeloid differentiated HL-60 cells. Cells were incubated in HBSS with or without 100 nM LTB_4 for 5 min at 37°C followed by [^{3}H] fMLP binding assay on ice.

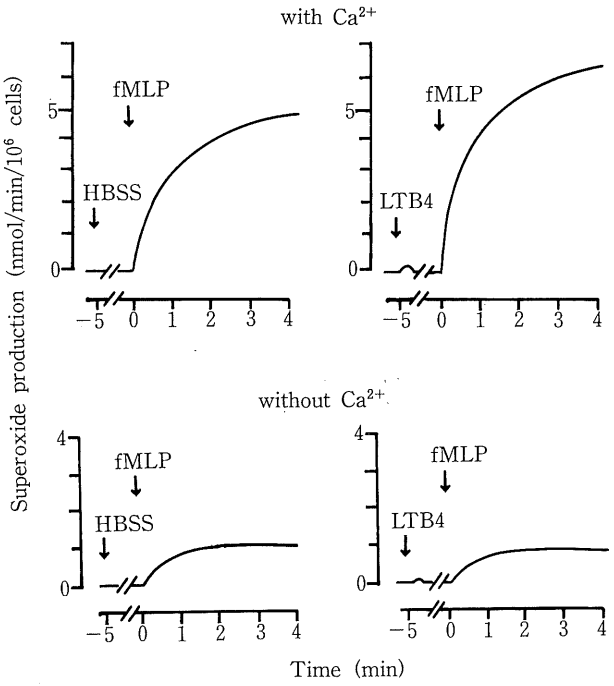

Fig. 2. Effect of elimination of extracellular Ca^{2+} on LTB_4-induced enhancement of superoxide production evoked by fMLP. Myeloid differentiated HL-60 cells were preincubated with 100 nM LTB_4 or HBSS for 5 min before addition of 100 nM fMLP. Cells were incubated in HBSS containing Ca^{2+} (upper figures) or in Ca^{2+}-free HBSS containing 1 mM EGTA (lower figures).

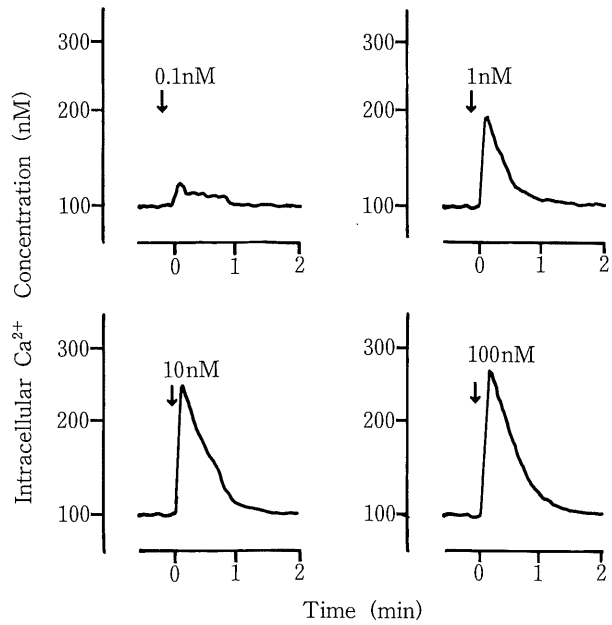

Fig. 3. LTB_4-induced intracellular Ca^{2+} mobilization of myeloid differentiated HL-60 cells. Maximal level of intracellular Ca^{2+} mobilization of the cells stimulated with 0.1, 1, 10 and 100 nM of LTB_4 was 123 ± 25.3 nM, 194.4 ± 7.8 nM, 245.2 ± 16.5 nM and 248.2 ± 19.7 nM, respectively. Base-line intracellular Ca^{2+} was 113 ± 8.5 nM. Triplicate determinations were performed in each condition.

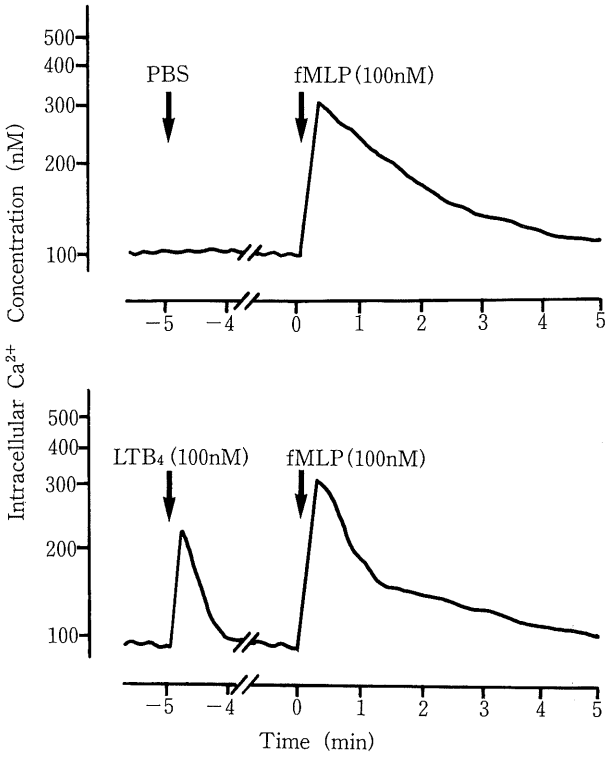

Fig. 4. Effect of LTB_4 on fMLP-induced intracellular Ca^{2+} mobilization in myeloid differentiated HL-60 cells. The decline of the transient overshoot is presented as tangent D, where D is the angle between the base line and the tangential line of the decreasing phase of transient intracellular Ca^{2+} mobilization that draws from the peak point to a level at 30 sec later. Tangent D of the decreasing phase of intracellular Ca^{2+} in the cells pretreated with PBS or 100 nM LTB_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^{2+}, LTB_4 significantly enhanced fMLP-induced superoxide production in Ca^{2+} containing HBSS.

LTB_4 itself induced intracellular Ca^{2+} rise in a dose dependent manner (Fig. 3). EC_{50} for LTB_4 to increase intracellular Ca^{2+} level in myeloid differentiated HL-60 cells was 0.78 nM, which was also approximately similar to K_{dH} for LTB_4.

However, fMLP-induced intracellular Ca^{2+} rise was not affected by LTB_4 pretreatment (Fig. 4). The maximal level of increase of 100 nM fMLP-induced intracellular Ca^{2+} in 100 nM LTB_4-pretreated cells (332.6 ± 49.3 nM) was similar to that of non-pretreated ones (330.7 ± 65.9 nM). When LTB_4 (100 nM)-pretreated cells were stimulated with 1 nM and 10 nM fMLP, the maximal level of intracellular Ca^{2+} was also similar to that of the non-pretreated ones (data not shown). The transient overshoot in intracellular Ca^{2+} induced by fMLP in LTB_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[8,10,27,28]. 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[13], 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[21]. 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[26]. In myeloid differentiated HL-60 cells, inositol trisphosphate is thought to be a signal for fMLP-induced Ca^{2+} mobilization[3]. 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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