Exogenous Leukotriene B₄ (LTB₄) Inhibits Human Neutrophil Generation of LTB₄ from Endogenous Arachidonic Acid During Opsonized Zymosan Phagocytosis¹

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ABSTRACT

The effect of exogenous leukotriene B₄ (LTB₄) on opsonized zymosan-stimulated human neutrophil formation of 5-lipox
genase products and arachidonic acid release was directly assessed using reverse-phase HPLC/tandem mass spectrome

tic methods for quantitation. Stable isotopically labeled LTB₄, [1,2-¹³C₂]LTB₄, caused a dose-dependent inhibition of

LTB₄ production in isolated human neutrophils with significant inhibition (60 ± 7% of control levels) when 0.12 nM [¹³C₂]LTB₄

was present. Production of 5-hydroxy-6,8,11,14-eicosatetraenoic acid and release of free arachidonic acid were also dose-
dependently inhibited by exogenous LTB₄. Metabolites of LTB₄, 20-hydroxy-LTB₄ and 3(S)-hydroxy-LTB₄, also significa
tantly reduced LTB₄ production to levels as low as 10 ± 6% and 10 ± 7% of control levels, respectively, when present exogenously at 10 nM. Exogenous 5-hydroxy-6,8,11,14-eicosatetraenoic acid at concentrations as high as 10 nM produced no significant reduction in LTB₄ biosynthesis during zymosan-stimulated human neutrophil production of LTB₄. The inhibitory effect of LTB₄ could be partially reversed by the LTB₄ receptor antagonist U 75302. Furthermore, an alternative stimulus, N-
formyl-methionyl-leucyl-phenylalanine (100 nM), did not inhibit the production of LTB₄ in opsonized zymosan-stimulated hu

man neutrophils. These results suggest that activation of the LTB₄ receptor on the human neutrophil during phagocytosis limits the ultimate biosynthesis of LTB₄. This autocrine effect is opposite to that observed when neutrophils have much of the signal transduction pathways bypassed when stimulated with calcium ionophore A23187 or treated with exogenous free arachidonic acid.

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ABBREVIATIONS: LTB₄, leukotriene B₄; LC/MS/MS, reverse-phase HPLC tandem mass spectrometry; 5-HETE, 5-hydroxy-6,8,11,14-eicosatetraenoic acid; 20-OH-LTB₄, 20-hydroxy-LTB₄; fMLP, N-formyl-methionyl-leucyl-phenylalanine; LTA₄, leukotriene A₄; 5-HpETE, 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid; cPLA₂, cytosolic phospholipase A₂; BSA, bovine serum albumin; HBSS, Hank’s buffered saline solution; PMN, polymorphonuclear leukocyte; PFB, pentafluorobenzyl ester; MRM, multiple reaction monitoring.
sis could be regulated by LTB₄ itself (McDonald et al., 1992; McDonald et al., 1994). Recent studies have suggested that free arachidonic acid as well as products of 5-lipoxygenase may act to increase the activity of phospholipase A₂ in the human neutrophil. In ionophore-stimulated cells, 5(S)-HETE was found to potentiate markedly the release of arachidonic acid in human neutrophils and to increase the biosynthesis of 5(S)-HETE and LTB₄ (Billah et al., 1985). Exogenous arachidonic acid and LTB₄ were reported to have similar effects when added to cells before they were stimulated with calcium ionophore, which suggests that increased phospholipase A₂ activity was a result of the addition of these exogenous substrates. An additional study has shown that the activity of the 85-kDa cytosolic PLA₂ isolated from human neutrophils shown to have unique specificity for arachidonic acid was increased 2-fold when cells were exposed to 5-HETE, LTB₄ or free arachidonic acid (Wijkander et al., 1995). It has been proposed that the increase in cPLA₂ activity initiated by exogenous free arachidonic acid results from conversion of this arachidonic acid into LTB₄, because the increase in activity initiated by arachidonic acid could be blocked by treatment of the neutrophils with inhibitors of 5-lipoxygenase (Lew et al., 1991).

Separate studies have suggested that LTB₄ activates human neutrophil 5-lipoxygenase indirectly, probably through signal transduction pathways. Incubation of cells with stable isotopically labeled LTB₄ and arachidonic acid resulted in significant increases in LTB₄ biosynthesis and the synthesis of 5,15-diHETE from 15-HpETE (McDonald et al., 1992). In these studies, a LTB₄ receptor antagonist reduced LTB₄ biosynthesis. The calcium ionophore in the presence of exogenous LTB₄ was found to increase 5-lipoxygenase translocation to the nuclear fraction of neutrophils (Serio et al., 1997), adding further support to the idea that LTB₄ may stimulate its own biosynthesis via the membrane LTB₄ receptor.

We have recently developed a mass spectrometry-based method for the simultaneous quantitation of LTB₄, ω-oxidized metabolites of LTB₄ and 5-HETE in order to assess multiple 5-lipoxygenase products in a single analytical determination (Wheelan and Murphy, 1997). This method has detection limits in the picogram level and so makes it possible to study leukotriene biosynthesis under physiologically relevant conditions where the absolute quantity of LTB₄ produced is below detection limits that typically apply when we use HPLC with UV detection. In addition, the ability to characterize exogenously added LTB₄ uniquely as a carbon-13-labeled analog two atomic mass units heavier than endogenously produced LTB₄ made possible the direct assessment of newly synthesized LTB₄ from endogenous arachidonic acid in the presence of exogenous [13C₂]LTB₄. With this approach, exogenous LTB₄ at concentrations as low as 0.1 nM was found to inhibit significantly the opsonized zymosan-stimulated production of LTB₄ by human neutrophils. Furthermore, the release of free arachidonic acid was found to be inhibited by exogenous LTB₄, which suggests that both responses may be under LTB₄ receptor control and linked to unique signaling events initiated by phagocytosis.

Materials and Methods

Materials. The following drugs and chemicals were kindly provided by or obtained from the sources indicated: LTB₄, [6,7,14,15,16,17,19,20-3H]LTB₄, [5,6,8,9,11,12,14,15-d₄]S-hydroxy-6,8,11,14-eicosatetraenoic acid (d₄-5-HETE), 5(S)-HETE, 20-OOH-LTB₄ and [5,6,8,9,11,12,14,15-d₅]-arachidonic acid (d₅-AA) (Cayman Chemical Co., Ann Arbor, MI), U 75302 (Biomol Research Laboratories, Plymouth, PA), zymosan A and [1,2-13C₂]LTB₄ (Sigma Chemical Co., St. Louis, MO), 3(S)-OH-LTB₄ (Dr. J.R. Falck (Wheelan et al., 1994)), fMLP (Vega Biotechnologies Inc., Tucson, AZ) and the calcium ionophore A23187 (Calbiochem, La Jolla, CA). The chemical purity and structural identity of all synthetic LTB₄ isotopomers were checked by UV spectroscopy, HPLC and mass spectrometry. All solvents were HPLC grade obtained from Fisher Scientific (Fair Lawn, NJ), and other commercially available reagents were the highest purity available.

Cell incubation. Human PMNs were prepared as previously described (Lynch and Henson, 1986). Substrates, in ethanol, were evaporated to dryness under nitrogen and redisolved in HBSS (900 μl containing 0.05% (w/v) BSA). The LTB₄ receptor inhibitor and fMLP were dissolved in DMSO that was added directly to cell suspensions. Solutions were added to pelleted cells (18 × 10⁶ neutrophils), and the cells were gently resuspended. For inhibitor studies, cells were preincubated at 37°C for 5 min with the concentrations of inhibitors presented in the text. A suspension of opsonized zymosan (10 mg in 100 μl of HBSS/0.05% BSA), prepared as previously described (Lynch and Henson, 1986), was added to the cells, which were then incubated at 37°C for an additional 10 min. Four volumes of cold ethanol were next added with addition of internal standards, d₁₄-LTB₄, d₁₅-5-HETE and d₁₅-arachidonic acid (10 ng for each internal standard). The samples were kept at 0°C for 1 h and then centrifuged. The supernatants were decanted and evaporated to near dryness under a flow of dry nitrogen. Samples were reconstituted in 15% methanol (2 ml), and metabolites were purified by solid-phase extraction as previously described (Wheelan and Murphy, 1995). Purified samples, in methanol (2 ml), were stored at <0°C until analyzed.

Free arachidonate quantitation. Aliquots of each sample (400 ml) were dried under nitrogen and arachidonic acid derivatized to the PFB ester by the addition of N,N-diisopropylethylamine (10%, v/v) in acetonitrile (50 μl) and pentafluorobenzyl bromide (10%, v/v) in acetonitrile (50 μl). Samples were kept at room temperature for 30 min, after which volatile reagents and solvents were removed by evaporation under nitrogen. Acetonitrile (20 μl) was added to the dried samples. Quantitation of arachidonic acid was performed by gas chromatography/mass spectrometric analysis in the negative ion mode (Hadley et al., 1988). The mass spectrometer (Finnigan SSQ70, San Jose, CA) employed methane as the moderating gas. Samples (1 μl) were introduced into the mass spectrometer by a 5 m × 0.25 mm DB-1 GC capillary column (J & W Scientific, Folsom, CA). Initial column temperature of 150°C was used followed by a linear gradient of 15°C/min to 300°C. The injector temperature was maintained at 275°C and the transfer line at 300°C. The mass spectrometer was operated in the selected-ion-monitoring (SIM) mode, monitoring ions at m/z 303 for the [M-PFB⁺] ion of arachidonic acid and m/z 311 for the [M-PFB⁺]²⁻ ion of the d₁₅-arachidonic acid internal standard.

Neutrophil response. The extent of neutrophil phagocytosis was assessed in separate experiments, where cells were incubated as described above and, at the end of the incubation period, a 50-μl aliquot was added to 100 μl of cytosin buffer containing 2 mM EDTA. The cell suspension was centrifuged for 2 min at 1000 g onto a glass slide. Attached cells were fixed and stained using Difco Manual, after which volatile reagents and solvents were removed by evaporation under nitrogen. Attached cells were viewed at 100× magnification with Leica fluorescence microscope. The extent of neutrophil phagocytosis was assessed in separate experiments, where cells were incubated as described above and, at the end of the incubation period, a 50-μl aliquot was added to 100 μl of cytosin buffer containing 2 mM EDTA. The cell suspension was centrifuged for 2 min at 1000 g onto a glass slide. Attached cells were fixed and stained using Difco Fluorescent Dyes (10 mg in 100 ml), containing 0.05% (w/v) BSA. The LTB₄ receptor inhibitor and fMLP were dissolved in DMSO that was added directly to cell suspensions. Solutions were added to pelleted cells (18 × 10⁶ neutrophils), and the cells were gently resuspended. For inhibitor studies, cells were preincubated at 37°C for 5 min with the concentrations of inhibitors presented in the text. A suspension of opsonized zymosan (10 mg in 100 μl of HBSS/0.05% BSA), prepared as previously described (Lynch and Henson, 1986), was added to the cells, which were then incubated at 37°C for an additional 10 min. Four volumes of cold ethanol were next added with addition of internal standards, d₁₄-LTB₄, d₁₅-5-HETE and d₁₅-arachidonic acid (10 ng for each internal standard). The samples were kept at 0°C for 1 h and then centrifuged. The supernatants were decanted and evaporated to near dryness under a flow of dry nitrogen. Samples were reconstituted in 15% methanol (2 ml), and metabolites were purified by solid-phase extraction as previously described (Wheelan and Murphy, 1995). Purified samples, in methanol (2 ml), were stored at <0°C until analyzed.

Free arachidonate quantitation. Aliquots of each sample (400 ml) were dried under nitrogen and arachidonic acid derivatized to the PFB ester by the addition of N,N-diisopropylethylamine (10%, v/v) in acetonitrile (50 μl) and pentafluorobenzyl bromide (10%, v/v) in acetonitrile (50 μl). Samples were kept at room temperature for 30 min, after which volatile reagents and solvents were removed by evaporation under nitrogen. Acetonitrile (20 μl) was added to the dried samples. Quantitation of arachidonic acid was performed by gas chromatography/mass spectrometric analysis in the negative ion mode (Hadley et al., 1988). The mass spectrometer (Finnigan SSQ70, San Jose, CA) employed methane as the moderating gas. Samples (1 μl) were introduced into the mass spectrometer by a 5 m × 0.25 mm DB-1 GC capillary column (J & W Scientific, Folsom, CA). Initial column temperature of 150°C was used followed by a linear gradient of 15°C/min to 300°C. The injector temperature was maintained at 275°C and the transfer line at 300°C. The mass spectrometer was operated in the selected-ion-monitoring (SIM) mode, monitoring ions at m/z 303 for the [M-PFB⁺] ion of arachidonic acid and m/z 311 for the [M-PFB⁺]²⁻ ion of the d₁₅-arachidonic acid internal standard.
scribed (Powell et al., 1993), with minor changes. Actual calcium levels were calculated as described (Grynkiewicz et al., 1985).

Quantitation of LTB₄ o-oxidized LTB₄ metabolites and 5-HETE. Quantitation of eicosanoids was performed using a LC/MS/MS method that permitted quantitation at the low pg/ml concentration range (Wheelan and Murphy, 1997). Briefly, samples were dried under nitrogen and reconstituted in methanol (100 µl). Just before analysis, water (300 µl) containing 0.05% acetic acid with pH adjusted to 5.0 using ammonium hydroxide (mobile phase A) was added. Samples (50 µl) were analyzed by reverse-phase HPLC with a 50-µl sample loop and an Ultrasphere column (1.00 × 150 mm, 3 µm C18; Phenomenex, Rancho Palos Verdes, CA) at a flow rate of 50 µl/min. A linear gradient from 30% B (acetonitrile/methanol, 65:35) to 100% B over 20 min was used. This reverse-phase HPLC system resulted in complete separation of LTB₄ and all stereoisomers of LTB₄. Column effluent was introduced into the mass spectrometer by a 0.5 m × 50 mm fused silica capillary. MS/MS analyses were performed on a Sciex API III⁺ triple quadrupole (PE Sciex, Thornhill, Ontario, Canada) in the negative ion mode by using MRM and monitoring the transitions of the molecular anion of each eicosanoid to the major fragment ion (Wheelan et al., 1996). The transitions monitored were m/z 335 → 195 for LTB₄, 337 → 197 for [1,2-¹³C₂]LTB₄, m/z 339 → 197 for d₄-LTB₄, m/z 351 → 195 for 20-OH-LTB₄, 353 → 197 for 20-OH-[¹³C₂]LTB₄, m/z 365 → 195 for 20-COOH-LTB₄, m/z 319 → 115 for 5-HETE and m/z 327 → 116 for d₄-5-HETE. A dwell time of 400 msec for each transition was used and resulted in a total scan time of 3.6 sec. Argon was used in the collision cell at a pressure equivalent to 200 × 10⁻¹³ molecules/cm². The spray voltage was −3400 V and the orifice voltage was −60 V with a collisional offset voltage of 20 eV. Peak areas and calibration curves were obtained using the Sciex Marquaq program.

Statistical analysis. Data were analyzed using a directional Student’s t test. Mean responses were compared individually with control levels. Differences were considered significant when P < .025 and highly significant when P < .001.

Results

Stimulation of human polymorphonuclear leukocytes (1.8 × 10⁷ cells/ml) with oposizenzymed LTB₄ (100 mg/ml) resulted in the production of 2.1 ± 0.2 ng of LTB₄/10⁷ cells (n = 4, duplicate samples) and a concentration at the end of the incubation of 6 nM LTB₄. The oxidized metabolites of LTB₄, 20-OH-LTB₄ and 20-COOH-LTB₄, were produced in similar quantities at 1.9 ± 0.3 ng/10⁷ cells and 1.6 ± 0.5 ng/10⁷ cells, respectively. The production of 5-HETE was measured at 3.1 ± 0.5 ng/10⁷ cells, which did not include the possible formation of 5-HETE that may have been subsequently esterified into phospholipids (Richards et al., 1986). A typical MRM profile is shown in figure 1A, where the signal from the transition m/z 335 → 195 was used to quantitate LTB₄ derived from endogenous arachidonic acid by comparison with the signal (transition m/z 339 → 197) derived from the internal standard, d₄-LTB₄.

When [¹³C₂]LTB₄, in concentrations observed in this phagocytosis model, was added to neutrophil incubations immediately before oposizenzymed zymosan stimulation, a striking inhibition of LTB₄ biosynthesis from endogenous arachidonate was observed. Addition of [¹³C₂]LTB₄ at 0.12 nM (40 pg/ml) at the time of stimulation (fig. 1B) resulted in formation of only 54% of the amount of LTB₄ produced in the absence of added exogenous [¹³C₂]LTB₄ (fig. 1A). When the concentration of [¹³C₂]LTB₄ was increased 100-fold to 12 nM (4 ng/ml), production of LTB₄ was reduced to only 2% (fig. 1C) of that produced in the absence of exogenous LTB₄. In these studies, the transition m/z 337 → 197 was used to verify the quantity of exogenous [¹³C₂]LTB₄ added to the cells before stimulus (data not shown). In a series of experiments (n = 4), the production of LTB₄ in the presence of 0.001 nM [¹³C₂]LTB₄ was not significantly changed from that measured in control incubations, but it was reduced to 60 ± 7% in the presence of 0.12 nM [¹³C₂]LTB₄ and was reduced to only 3 ± 2% in the presence of 12 nM [¹³C₂]LTB₄ (fig. 2A). The production of the oxidized LTB₄ metabolites 20-hydroxy-LTB₄ and 20-carboxyl-LTB₄, and that of 5-HETE, were similarly affected by the addition of exogenous LTB₄ during the simultaneous addition of oposizenzymed LTB₄ to neutrophil preparation.

Exogenously added LTB₄ was also found to inhibit dose-dependently the release of free arachidonic acid, but the
the presence of 12 nM [13C2]LTB4 (fig. 2).

A dose-dependent inhibition of 5-lipoxygenase products formed during phagocytosis and of the release of free arachidonic acid was observed when [13C2]LTB4 was added 5 min before stimulation with opsonized zymosan (fig. 2B). The inhibitory effect of exogenous LTB4 at 0.12 nM was somewhat less what it was added before stimulation than when it was added at the same time as opsonized zymosan. The inhibition of LTB4, of its ω-oxidized metabolites and of 5-HETE production caused by exogenous LTB4 at 12 nM was less when it was added 5 min before stimulation (45 ± 4% and 62 ± 7%, respectively) when compared with values obtained without preincubation.

Identical results for the inhibition of 5-lipoxygenase-derived eicosanoids and free arachidonic acid were observed when exogenous LTB4 was added and when the [5,6,14,15-d4]LTB4 stable isotopically labeled analog was added, and the quantitation of endogenous LTB4 was carried out using [13C2]LTB4 as the added internal standard (data not shown).

Formation of 20-OH[13C2]LTB4 derived from the exogenously added LTB4 was also quantitated by monitoring the mass spectrometric transition m/z 353 → 197 as well as unmetabolized [13C2]LTB4, which was detected by the transition m/z 337 → 197. In comparison, the production and metabolism of endogenously produced LTB4 in the absence of exogenous LTB4 revealed a ratio of LTB4 to 20-OH-LTB4 corresponding to 1.1. In contrast, the ratio of [13C2]LTB4 to 20-OH[13C2]LTB4 when [13C2]LTB4 was added to the neutrophil preparation at 12 nM, was found to be 6.5 ± 2.4. These results revealed that endogenously produced LTB4 was more extensively metabolized than exogenously added LTB4 during the incubation period.

Oposonized zymosan-stimulated production of LTB4 was also inhibited by treatment with exogenous 20-OH-LTB4 (10 nM) and 3-OH-LTB4 (10 nM), where the level of LTB4 production was 10 ± 6% and 10 ± 7%, respectively, of control values (fig. 3A). This inhibiting effect was not so potent as that of LTB4 at this concentration, as expected for the potency of these LTB4 metabolites (Wheelan et al., 1994). When 20-OH-LTB4 (10 nM) and 3-OH-LTB4 (10 nM) were added 5 min before stimulation with opsonized zymosan, LTB4 production was 65 ± 19% and 32 ± 11% of control values, respectively (fig. 3B). In contrast, the addition of 5-HETE (10 nM) at the time of stimulation resulted in a slightly increased production of LTB4 (108 ± 4%) and had no significant effect when added 5 min before stimulation.

The inhibition of LTB4 production caused by exogenous [13C2]LTB4 (12 nM) was reversed in a dose-dependent manner when neutrophils were incubated with the LTB4 receptor antagonist U 75302. A maximum effect was reached at 10 μM U 75302 (65 ± 25% of control levels) (fig. 4). However, at higher concentrations of the inhibitor, the amount of endogenous LTB4 synthesized did not further increase, and at the highest concentration of U 75302 (100 μM), there was no significant difference from the amount of LTB4 produced by opsonized zymosan-treated and [13C2]LTB4-treated neutrophils. Also, the production of endogenous LTB4 was significantly reduced from control levels (opsonized zymosan only) when neutrophils were preincubated for 10 min with U 75302 (10 μM) followed by opsonized zymosan (data not shown). Additional experiments with this inhibitor revealed that U 75302 was also an agonist for the human neutrophil. When it was added to human neutrophils at 1.4 μM, intracellular free calcium ion levels rose to 55% of the response induced by 2

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Fig. 2. Inhibition of the formation of LTB4 synthesized by isolated human neutrophils after treatment with opsonized zymosan. Exogenous [13C2]LTB4 was added at three concentrations, and the production of LTB4, that of 5-HETE and the release of arachidonic acid were measured after a 10-min stimulation. A) Exogenous LTB4 was added to the isolated neutrophils simultaneously with the opsonized zymosan. B) Addition of exogenous [13C2]LTB4 5 min before the addition of opsonized zymosan to isolated human neutrophils. Values are the average of four separate human neutrophil preparations. The same preparation of opsonized zymosan was used, and samples were analyzed in duplicate. Error bars indicate the S.E.M., and the level of significance is indicated as the number of asterisks with significant difference from control values (P < .025, *) and highly significant difference (P < .001, **).
sions at the time of opsonized zymosan stimulation had no significant effect on the biosynthesis of LTB₄ and increased LTB₄ levels to 166 ± 36% when 1 µM was added (table 1).

In order to test whether exogenous LTB₄ altered the phagocytic process itself, we carried separate experiments out to ascertain whether the number of zymosan particles ingested per neutrophil or the number of neutrophils undergoing phagocytosis was altered. Approximately 80% of control as well as [¹³C₂]LTB₄-pretreated neutrophils underwent phagocytosis and could not be distinguished from one another. The control phagocytosing neutrophils had 11.6 ± 1.5 (S.E., n = 7) particles per cell, and LTB₄-pretreated neutrophils had 11.0 ± 1.4 (S.E., n = 7) particles per cell.

**Discussion**

A large number of agonists are known to initiate the 5-lipoxygenase pathway of arachidonic metabolism leading to production of LTA₄ within the human neutrophil. A major product of subsequent LTA₄ metabolism is the chemotactic factor LTB₄, produced either by neutrophil (Borgeat and Samuelsson, 1979) or accessory cell (McGee and Fitzpatrick, 1985) LTA₄ hydrolase. A recent appreciation of the transcellular metabolism of LTA₄ has led to a consideration of a possible autocrine effect of LTB₄ on the neutrophil biosynthetic events. Activation of the G protein-linked LTB₄ receptor (Yokomizo et al., 1997) by concentrations of LTB₄ as low as 0.3 to 1 nM can lead to a significant elevation of intracellular calcium, a critical element required for 5-lipoxygenase translocation. Several investigations have provided evidence for a positive-feedback regulation of LTB₄ synthesis within a neutrophil via exogenous LTB₄ (McDonald et al., 1992, 1994) and the calcium ionophore (McDonald et al., 1994), exogenous arachidonic acid (Wijkander et al., 1995) or an antagonist of the LTB₄ receptor (McDonald et al., 1992; McDonald et al., 1994; Serio et al., 1997). These studies have used concentrations of exogenous 5-lipoxygenase products and free arachidonic acid in excess of the expected in vivo concentrations or have bypassed to some extent receptor-mediated intracellular signaling interactions through the use of the calcium ionophore. Although such model systems provide insight into the potential complexity of signaling events leading to activation of 5-lipoxygenase and release of free arachidonic acid, and leading ultimately to LTA₄ biosynthesis, we felt that physiologically relevant stimuli should be

**TABLE 1**

Effect of exogenous [¹³C₂]LTB₄ and fMLP on LTB₄ synthesis from endogenous arachidonic acid after stimulation of isolated human neutrophils with opsonized zymosan

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LTB₄ Produced (pmol LTB₄/10⁷ PMN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (100 nM fMLP)</td>
<td>0.65 ± 0.21*</td>
</tr>
<tr>
<td>Opsonized zymosan</td>
<td>12.68 ± 3.33</td>
</tr>
<tr>
<td>Opsonized zymosan + [¹³C₂]LTB₄ (10 nM)</td>
<td>2.23 ± 1.16</td>
</tr>
<tr>
<td>Opsonized zymosan + fMLP (100 nM)</td>
<td>12.95 ± 4.64</td>
</tr>
<tr>
<td>Opsonized zymosan + fMLP (1 µM)</td>
<td>19.36 ± 6.34</td>
</tr>
</tbody>
</table>

*Average of four neutrophil preparations with S.E.M. The same preparation of opsonized zymosan was used in these experiments.

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**Fig. 3.** Inhibition of LTB₄ biosynthesis after treatment of isolated human neutrophils with opsonized zymosan by 5(S)-HETE (10 nM), 20-OH-LTB₄ (10 nM) and 3(S)-OH-LTB₄ (10 nM) added at the time of stimulation with opsonized zymosan (n = 3; panel A) and added 5 min before the stimulation of neutrophils (n = 3; panel B). The error bars indicate S.E.M., and significance is indicated as described in figure 2.

**Fig. 4.** The effect of U 75302 on opsonized zymosan-stimulated cells treated with exogenous [¹³C₂]LTB₄. Control levels of LTB₄ synthesis with this preparation of opsonized zymosan resulted in 2.1 ng/10⁷ neutrophils without the addition of exogenous LTB₄. The resulting LTB₄ production in stimulated neutrophils was expressed as the percentage of control levels when cells were preincubated for 10 min and U 75302 (0.3–100 µM) and [¹³C₂]LTB₄ (10 nM) were added simultaneously with opsonized zymosan. The maximum amount of DMSO added to the cell incubation was 3 µl for the highest concentration of U 75302. Error bars indicate the S.E.M. (n = 3), and the level of significance is indicated as described in figure 2.

At 0.14 µM U 75302, intracellular calcium increased to 30% of that induced by 2 nM LTB₄.

The addition of fMLP (100 nM) to the neutrophil suspen-
studied in detail to assess whether a positive-feedback effect of exogenous LT$_B_4$ could be demonstrated.

Opsonized zymosan was used in these studies because it is a sufficient stimulus to initiate LT$_B_4$ biosynthesis (Claesson et al., 1981). The results obtained with opsonized zymosan and relevant concentrations of exogenous LT$_B_4$ suggest that the LT$_B_4$ receptor can regulate production of LT$_B_4$ in the phagocytizing neutrophil but that it does so in a negative-feedback manner. The quantitative measurement of LT$_B_4$ levels derived from endogenous arachidonate (corresponding to unlabeled LT$_B_4$) and exogenous LT$_B_4$ (corresponding to [13C$_2$]LT$_B_4$) was made possible by using a third LT$_B_4$ as internal standard ([D$_2$]LT$_B_4$) in a mass spectrometric assay. The results of these investigations revealed a significant inhibition of LT$_B_4$ biosynthesis with concentrations as low as 0.12 nM exogenous LT$_B_4$.

Although these results are not consistent with previous studies of a positive autocrine effect of LT$_B_4$, we were able to confirm in our neutrophil preparations the previously published reports that exogenous LT$_B_4$ moderately stimulates neutrophil LT$_B_4$ biosynthesis initiated by A23187 (data not shown). We also observed inhibition of zymosan-stimulated LT$_B_4$ biosynthesis with an LT$_B_4$ receptor antagonist. Taken together, these data suggest that regulation of LT$_B_4$ biosynthesis under conditions similar to those expected during phagocytosis is more complex than had been thought and that previously formed LT$_B_4$ (exogenous LT$_B_4$) may serve as a feedback inhibitor, probably through complex interactions of signal transduction pathways originating from the LT$_B_4$ receptor as well as the multiple receptors stimulated by zymosan that converge on LT$_A_4$ production. We observed that activation by fMLP, which works through a separate G protein-linked receptor, did not inhibit LT$_B_4$ production, which suggests a unique signal transduction pathway from the LT$_B_4$ receptor. Further support for the importance of the simultaneous interaction of two signal transduction pathways to inhibit LT$_B_4$ came from a reduction of the inhibiting effect of exogenous LT$_B_4$ when it was added to opsonized zymosan not simultaneously with the initiation of phagocytosis but 5 min before (Figs. 3 and 4).

The dose-dependent inhibition of LT$_B_4$ synthesis in opsonized zymosan-stimulated human neutrophils by exogenous LT$_B_4$ was quantitatively different from the dose-dependent inhibition of arachidonic acid release. This may indicate that the inhibition of 5-lipoxygenase products involves factors other than PLA$_2$ inhibition. These findings would be consistent with a recent study showing phosphorylation of cPLA$_2$ and increased cPLA activity in the isolated enzyme after exposure of cells to arachidonic acid, LT$_B_4$, or 5-HETE (Billah et al., 1985). In the intact cell, other factors may be operating that limit phospholipase activity or limit formation of 5-lipoxygenase products.

It has previously been shown that exogenous LT$_B_4$ at concentrations up to 10 nM had no effect on opsonized zymosan-stimulated production of superoxide in the human neutrophil (Gay et al., 1984). Our studies found no effect of exogenous LT$_B_4$ on zymosan phagocytosis. These observations suggested that the dose-dependent inhibition of LT$_B_4$ synthesis by exogenous LT$_B_4$ shown in the present study is not due to a nonspecific cellular inactivation.

Inhibition of LT$_B_4$ synthesis by exogenous LT$_B_4$ probably involved LT$_B_4$ receptor signaling events through the recently described G protein-coupled LT$_B_4$ receptor (Yokomizo et al., 1997). The LT$_B_4$-derived metabolites 20-OH-LT$_B_4$ and 3-OH-LT$_B_4$ also exert their effects on neutrophil function through the LT$_B_4$ receptor (Powell et al., 1996; Shirley et al., 1992), and they also inhibited LT$_B_4$ production in this model. In contrast, the 5-lipoxygenase metabolite 5(S)-HETE, which operates through a distinct receptor (O’Flaherty and Rossi, 1993), had no effect on LT$_B_4$ synthesis at doses relevant to its production. Partial reversal of exogenous LT$_B_4$ inhibition by the specific LT$_B_4$ receptor antagonist U 75302 (Lin et al., 1988) at low doses was also consistent with LT$_B_4$ receptor-mediated inhibition. However, high concentrations of U 75302 strongly inhibited LT$_B_4$ biosynthesis. At these doses, the inhibitor alone was found significantly to reduce LT$_B_4$ synthesis in opsonized zymosan-treated neutrophils as well as to cause a dose-dependent release of intracellular calcium ions even at concentrations as low as 0.1 nM. These results suggest a possible direct agonist effect of this drug on the human neutrophil. Such an agonist effect of U 75302 on the chemotaxis of guinea pig eosinophils has previously been reported (Taylor et al., 1991).

The LC/MS/MS method also permitted quantitation of LT$_B_4$-derived metabolites and differentiation of endogenous 20-OH-LT$_B_4$ formation and the oxidation of exogenous [13C$_2$]-LT$_B_4$. In calcium ionophore-stimulated human neutrophils, the oxidation of LT$_B_4$ has been suggested to occur after reuptake of LT$_B_4$ from the extracellular media (Cluzel et al., 1989; Williams et al., 1985). Ionophore stimulation, which bypasses native cell-signaling events that result in cell stimulation, may also alter normal transport and metabolism of LT$_B_4$. It has been shown that most of the LT$_B_4$ synthesized by human neutrophils in response to zymosan is retained intracellularly (Williams et al., 1985), in contrast to the release of more than 90% of the LT$_A_4$ synthesized in response to calcium ionophore even when suboptimal concentrations of ionophore are employed (Sala et al., 1996). Relatively more 20-OH-LT$_B_4$-derived metabolites were formed than the oxidized LT$_B_4$ derived from [13C$_2$]-LT$_B_4$ after zymosan stimulation. While the inhibitory effect of 20-OH-LT$_B_4$ on LT$_B_4$ synthesis appeared comparable to that of LT$_B_4$ at the 10 nM concentration range, further metabolism to 20-carboxy-LT$_B_4$ is typically observed after ionophore stimulation (Lindgren et al., 1981). This may account for the much diminished LT$_B_4$ production from zymosan-stimulated human neutrophils as compared with maximally ionophore-stimulated cells. The present results suggest that an important control mechanism in the synthesis of LT$_B_4$ works to limit significantly the inflammatory response to phagocytic stimuli.

References


