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

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ABSTRACT

The effect of exogenous leukotriene B₄ (LTB₄) on opsonized zymosan-stimulated human neutrophil formation of 5-lipoxygenase products and arachidonic acid release was directly assessed using reverse-phase HPLC/tandem mass spectrometric 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 significantly 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 human 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 LT_{B\textsubscript{4}} 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 A\textsubscript{2} 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 LT_{B\textsubscript{4}} (Bilhah et al., 1985). Exogenous arachidonic acid and LT_{B\textsubscript{4}} were reported to have similar effects when added to cells before they were stimulated with calcium ionophore, which suggests that increased phospholipase A\textsubscript{2} activity was a result of the addition of these exogenous substrates. An additional study has shown that the activity of the 85-kDa cytosolic PLA\textsubscript{2} isolated from human neutrophils shown to have unique specificity for arachidonic acid was increased 2-fold when cells were exposed to 5-HETE, LT_{B\textsubscript{4}} or free arachidonic acid (Wijkander et al., 1995). It has been proposed that the increase in cPLA\textsubscript{2} activity initiated by exogenous free arachidonic acid results from conversion of this arachidonic acid into LT_{B\textsubscript{4}}, 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 LT_{B\textsubscript{4}} activates human neutrophil 5-lipoxygenase indirectly, probably through signal transduction pathways. Incubation of cells with stable isotopically labeled LT_{B\textsubscript{4}} and arachidonic acid resulted in significant increases in LT_{B\textsubscript{4}} biosynthesis and the synthesis of 5,15-diiHETE from 15-HpETE (McDonald et al., 1992). In these studies, a LT_{B\textsubscript{4}} receptor antagonist reduced LT_{B\textsubscript{4}} biosynthesis. The calcium ionophore in the presence of exogenous LT_{B\textsubscript{4}} was found to increase 5-lipoxygenase translocation to the nuclear fraction of neutrophils (Serio et al., 1997), adding further support to the idea that LT_{B\textsubscript{4}} may stimulate its own biosynthesis via the membrane LT_{B\textsubscript{4}} receptor.

We have recently developed a mass spectrometry-based method for the simultaneous quantitation of LT_{B\textsubscript{4}}, \omega-oxidized metabolites of LT_{B\textsubscript{4}} 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 LT_{B\textsubscript{4}} produced is below detection limits that typically apply when we use HPLC with UV detection. In addition, the ability to characterize exogenously added LT_{B\textsubscript{4}} uniquely as a carbon-13-labeled analog two atomic mass units heavier than endogenously produced LT_{B\textsubscript{4}} made possible the direct assessment of newly synthesized LT_{B\textsubscript{4}} from endogenous arachidonic acid in the presence of exogenous \textsuperscript{13}C_{2}LT_{B\textsubscript{4}}. With this approach, exogenous LT_{B\textsubscript{4}} at concentrations as low as 0.1 nM was found to inhibit significantly the opsonized zymosan-stimulated production of LT_{B\textsubscript{4}} by human neutrophils. Furthermore, the release of free arachidonic acid was found to be inhibited by exogenous LT_{B\textsubscript{4}}, which suggests that both responses may be under LT_{B\textsubscript{4}} 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: LT_{B\textsubscript{4}}, [6,7,14,15-\textsuperscript{d}_{3}]LT_{B\textsubscript{4}}, [5,6,8,9,11,12,14-15-d_{5}] 5(S)-hydroxy-6,8,11,14-eicosatetraenoic acid (d\textsubscript{5}-5-HETE), 5(S)-HETE, 20-OH-LT_{B\textsubscript{4}} and [5,6,8,9,11,12,14,15-d\textsubscript{5}]arachidonic acid (d\textsubscript{5}-AA) (Cayman Chemical Co., Ann Arbor, MI), U 75302 (Biomol Research Laboratories, Plymouth, PA), zymosan A and [1,2-\textsuperscript{13}C_{2}]LT_{B\textsubscript{4}} (Sigma Chemical Co., St. Louis, MO), 3(S)-OH-LT_{B\textsubscript{4}} (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 LT_{B\textsubscript{4}} isotopimers 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 redissolved in HBSS (900 μl) containing 0.05% (w/v) BSA. The LT_{B\textsubscript{4}} receptor inhibitor and fMLP were dissolved in DMSO that was added directly to cell suspensions. Solutions were added to pelleted cells (18 × 10\textsuperscript{6} 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, d\textsubscript{1}-LT_{B\textsubscript{4}}, d\textsubscript{3}-5-HETE and d\textsubscript{5}-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-diisoproplylethylamine (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 at 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\textsuperscript{-}]\textsuperscript{-} ion of arachidonic acid and m/z 311 for the [M-PFB\textsuperscript{-}]\textsuperscript{-} ion of the d\textsubscript{5}-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 quick and then were viewed at 100×. Phagocytosis was assessed in 10 fields that typically had 10 cells each, for a total of 100 cells. Percent of neutrophils containing one or more particles and average number of zymosan particles ingested per neutrophil were recorded.

Human neutrophils loaded with Indo-1 were incubated with either 2 mM LT_{B\textsubscript{4}} or varying concentrations of the LT_{B\textsubscript{4}} receptor antagonist U 75302. Changes in intracellular calcium levels were determined by measuring the fluorescence of Indo-1 as previously de-
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 x 150 mm, 3 m 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 µm x 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 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 x 10^13 molecules/cm^2. 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 MacQuan 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 x 10^7 cells/ml) with opsonized LTB4 (100 ng/ml) resulted in the production of 2.1 ± 0.2 ng of LTB4/10^7 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/10^7 cells and 1.6 ± 0.5 ng/10^7 cells, respectively. The production of 5-HETE was measured at 3.1 ± 0.5 ng/10^7 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 [13C]LTB4, in concentrations observed in this phagocytosis model, was added to neutrophil incubations immediately before opsonized 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 opsonized zymosan to neutrophil preparation.

Exogenously added LTB4 was also found to inhibit dose-dependently the release of free arachidonic acid, but the
A dose-dependent inhibition of 5-lipoxygenase products formed during phagocytosis and of the release of free arachidonic acid was observed when \([^{13}C_2]LTB_4\) was added 5 min before stimulation with opsonized zymosan (fig. 2B). The inhibitory effect of exogenous \(LTB_4\) at 0.12 nM was somewhat less when it was added before stimulation than when it was added at the same time as opsonized zymosan. The inhibition of \(LTB_4\), of its \(\omega\)-oxidized metabolites and of 5-HETE production caused by exogenous \(LTB_4\) 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 \(LTB_4\) was added and when the [5,6,14,15-d_4]LTB_4 stable isotopically labeled analog was added, and the quantitation of endogenous \(LTB_4\) was carried out using \([^{13}C_2]LTB_4\) as the added internal standard (data not shown).

Formation of 20-OH[\(^{13}C_2\)]LTB_4 derived from the exogenously added \(LTB_4\) was also quantitated by monitoring the mass spectrometric transition \(m/z\) 353 → 197 as well as unmetabolized \([^{13}C_2]LTB_4\), which was detected by the transition \(m/z\) 337 → 197. In comparison, the production and metabolism of endogenously produced \(LTB_4\) in the absence of exogenous \(LTB_4\) revealed a ratio of \(LTB_4\) to 20-OH-LTB_4 corresponding to 1.1. In contrast, the ratio of \([^{13}C_2]LTB_4\) to 20-OH[\(^{13}C_2\)]LTB_4 when \([^{13}C_2]LTB_4\) was added to the neutrophil preparation at 12 nM, was found to be 6.5 ± 2.4. These results revealed that endogenously produced \(LTB_4\) was more extensively metabolized than exogenously added \(LTB_4\) during the incubation period.

Opsonized zymosan-stimulated production of \(LTB_4\) was also inhibited by treatment with exogenous 20-OH-LTB_4 (10 nM) and 3-OH-LTB_4 (10 nM), where the level of \(LTB_4\) production was 10 ± 6% and 10 ± 7%, respectively, of control values (fig. 3A). This inhibiting effect was not so potent as that of \(LTB_4\) at this concentration, as expected for the potency of these \(LTB_4\) metabolites (Wheelan et al., 1994). When 20-OH-LTB_4 (10 nM) and 3-OH-LTB_4 (10 nM) were added 5 min before stimulation with opsonized zymosan, \(LTB_4\) 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 \(LTB_4\) (108 ± 4%) and had no significant effect when added 5 min before stimulation.

The inhibition of \(LTB_4\) production caused by exogenous \([^{13}C_2]LTB_4\) (12 nM) was reversed in a dose-dependent manner when neutrophils were incubated with the \(LTB_4\) receptor antagonist U 75302. A maximum effect was reached at 10 \(\mu\)M U 75302 (65 ± 25% of control levels) (fig. 4). However, at higher concentrations of the inhibitor, the amount of endogenous \(LTB_4\) synthesized did not further increase, and at the highest concentration of U 75302 (100 \(\mu\)M), there was no significant difference from the amount of \(LTB_4\) produced by opsonized zymosan-treated and \([^{13}C_2]LTB_4\)-treated neutrophils. Also, the production of endogenous \(LTB_4\) was significantly reduced from control levels (opsonized zymosan only) when neutrophils were preincubated for 10 min with U 75302 (10 \(\mu\)M) 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 \(\mu\)M, intracellular free calcium ion levels rose to 55% of the response induced by 2
sions at the time of opsonized zymosan stimulation had no significant effect on the biosynthesis of LTβ₄ and increased LTβ₄ levels to 166 ± 36% when 1 µM was added (table 1).

In order to test whether exogenous LTβ₄ 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 LTβ₄-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 arachidonate metabolism leading to production of LTA₄ within the human neutrophil. A major product of subsequent LTA₄ metabolism is the chemotactic factor LTβ₄, 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 LTβ₄ on the neutrophil biosynthetic events. Activation of the G protein-linked LTβ₄ receptor (Yokomizo et al., 1997) by concentrations of LTβ₄ 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 LTβ₄ synthesis within a neutrophil via exogenous LTβ₄ (McDonald et al., 1992; McDonald et al., 1994) and the calcium ionophore (McDonald et al., 1994), exogenous arachidonic acid (Wijkander et al., 1995) or an antagonist of the LTβ₄ 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 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 LTβ₄ synthesis from endogenous arachidonic acid after stimulation of isolated human neutrophils with opsonized zymosan

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LTβ₄ Produced (pmol LTβ₄/10⁷ 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>12.98 ± 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.
studied in detail to assess whether a positive-feedback effect of exogenous LTB4 could be demonstrated.

Opsonized zymosan was used in these studies because it is a sufficient stimulus to initiate LTB4 biosynthesis (Claesson, et al., 1981). The results obtained with opsonized zymosan and relevant concentrations of exogenous LTB4 suggest that the LTB4 receptor can regulate production of LTB4 in the phagocytizing neutrophil but that it does so in a negative-feedback manner. The quantitative measurement of LTB4 levels derived from endogenous arachidonate (corresponding to unlabeled LTB4) and exogenous LTB4 (corresponding to [13C2]-LTB4) was made possible by using a third LTB4 as internal standard ([1H]-LTB4) in a mass spectrometric assay. The results of these investigations revealed a significant inhibition of LTB4 biosynthesis with concentrations as low as 0.12 nM exogenous LTB4.

Although these results are not consistent with previous studies of a positive autocrine effect of LTB4, we were able to confirm in our neutrophil preparations the previously published reports that exogenous LTB4 moderately stimulates neutrophil LTB4 biosynthesis initiated by A23187 (data not shown). We also observed inhibition of zymosan-stimulated LTB4 biosynthesis with an LTB4 receptor antagonist. Taken together, these data suggest that regulation of LTB4 biosynthesis under conditions similar to those expected during phagocytosis is more complex than had been thought and that previously formed LTB4 (exogenous LTB4) may serve as a feedback inhibitor, probably through complex interactions of signal transduction pathways originating from the LTB4 receptor as well as the multiple receptors stimulated by zymosan that converge on LTA4 production. We observed that activation by fMLP, which works through a separate G protein-linked receptor, did not inhibit LTB4 production, which suggests a unique signal transduction pathway from the LTB4 receptor. Further support for the importance of the simultaneous interaction of two signal transduction pathways to inhibit LTB4 came from a reduction of the inhibiting effect of exogenous LTB4 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 LTB4 synthesis in opsonized zymosan-stimulated human neutrophils by exogenous LTB4 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 PLA2 inhibition. These findings would be consistent with a recent study showing phosphorylation of cPLA2 and increased cPLA2 activity in the isolated enzyme after exposure of cells to arachidonic acid, LTB4, 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 LTB4 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 LTB4 on zymosan phagocytosis. These observations suggested that the dose-dependent inhibition of LTB4 synthesis by exogenous LTB4 shown in the present study is not due to a nonspecific cellular inactivation.

Inhibition of LTB4 synthesis by exogenous LTB4 probably involved LTB4 receptor signaling events through the recently described G protein-coupled LTB4 receptor (Yokomizo et al., 1997). The LTB4-derived metabolites 20-OH-LTB4 and 3-OH-LTB4 also exert their effects on neutrophil function through the LTB4 receptor (Powell et al., 1996; Shirley et al., 1992), and they also inhibited LTB4 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 LTB4 synthesis at doses relevant to its production. Partial reversal of exogenous LTB4 inhibition by the specific LTB4 receptor antagonist U 75302 (Lin et al., 1988) at low doses was also consistent with LTB4 receptor-mediated inhibition. However, high concentrations of U 75302 strongly inhibited LTB4 biosynthesis. At these doses, the inhibitor alone was found significantly to reduce LTB4 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 μM. 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 LTB4-derived metabolites and differentiation of endogenous 20-OH-LTB4 formation and ω-oxidation of exogenous [13C2]-LTB4. In calcium ionophore-stimulated human neutrophils, ω-oxidation of LTB4 has been suggested to occur after reuptake of LTB4 from the extracellular media (Cluzel et al., 1989; Williams et al., 1985). Ionophore stimulation, which bypasses normal cell-signaling events that result in cell stimulation, may also alter normal transport and metabolism of LTB4. It has been shown that most of the LTB4 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 LTA4 synthesized in response to calcium ionophore even when suboptimal concentrations of ionophore are employed (Sala et al., 1996). Relatively more ω-oxidized LTB4-derived metabolites were formed than ω-oxidized LTB4 derived from [13C]-LTB4 after zymosan stimulation. While the inhibitory effect of 20-OH-LTB4 on LTB4 synthesis appeared comparable to that of LTB4 at the 10 nM concentration range, further metabolism to 20-carboxy-LTB4 was typically observed after ionophore stimulation (Lindgren et al., 1981). This may account for the much diminished LTB4 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 LTB4 works to limit significantly the inflammatory response to phagocytic stimuli.

References


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