Activation of the human neutrophil 5-lipoxygenase by leukotriene B₄

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1 In the present study, we demonstrate that leukotriene B₄ (LTB₄) has the ability to activate the human neutrophil 5-lipoxygenase (5-LO).

2 Stimulation of neutrophils with 30 nM 14,15-dideuterio-LTB₄ (D₂-LTB₄) failed to induce the synthesis of LTB₄ from endogenous arachidonic acid (AA), but stimulated the formation of LTB₄ from 3.3 µM exogenous AA, as determined by GC-MS analysis.

3 The stimulatory effect of LTB₄ on 5-LO activity was further examined with an alternative substrate; LTB₄ time- and dose-dependently stimulated the 5-LO-mediated conversion of exogenous 15(S)-hydroperoxy-5,8,11,13-(Z,Z,Z,E)-eicosatetraenoate (15-HpETE) into 5(S),15(S)-dihydroxy-6,8,11,13,15-(E,Z,Z,E)-eicosatetraenoate (5,15-DiHETE), with a threshold effect at 300 pM.

4 The ability of LTB₄ to activate the 5-LO showed structural specificity, since LTB₄ was found to be 100 times more potent than ω-hydroxy-LTB₄, and 300 times more potent than its Δ⁶-trans-12-epi-isomer.

5 The LTB₄-induced 5-LO activation was effectively inhibited by MK-886 (an inhibitor of 5-LO translocation), by pertussis toxin, and by the LTB₄ receptor antagonist, LY-223982.

6 These results demonstrate that the binding of LTB₄ to its cell-surface receptor results in 5-LO activation in a process mediated by pertussis toxin-sensitive guanine nucleotide-binding proteins. Our data also suggest that the underlying mechanism involves a translocation of the 5-LO to the membrane. These findings raise the possibility that LTB₄ produced by phagocytes may positively feedback on its own synthesis.

Keywords: Arachidonate 5-lipoxygenase; 5-lipoxygenase-activating protein; translocation; guanine nucleotide-binding proteins; receptors; inflammation

Introduction

Leukotriene B₄ (LTB₄), a 5-lipoxygenase (5-LO) metabolite of arachidonic acid (AA) (Borgeat & Samuelsson, 1979), is a potent stereospecific leukocyte activator which was originally characterized as an endogenous neutrophil chemotactic factor (Ford-Hutchinson, 1980). It has since been shown to stimulate many other neutrophil functions including degranulation, aggregation, adherence, and calcium mobilization (Naccache *et al.*, 1989a; Ford-Hutchinson, 1990). LTB₄ also acts upon other cells of the immune system, such as eosinophils, monocytes and lymphocytes (Rola-Pleczynski, 1985). Because of its well-characterized effects on various cells of the immune system, LTB₄ has become widely viewed as a potent mediator of inflammation and allergy.

Neutrophils and other phagocytes synthesize LTB₄ and related compounds in response to a variety of stimuli, notably, calcium ionophores, particulate agonists (such as urate and pyrophosphate crystals, as well as zymosan), and the soluble stimuli N-formyl-methionyl-leucyl-phenylalanine (fMLP), platelet-activating factor (PAF), interleukin-8 (IL-8) and complement component C5a (Borgeat & Samuelsson, 1979; Claesson *et al.*, 1981; Lin *et al.*, 1982; Clancy *et al.*, 1983; Poubelle *et al.*, 1987; Schröder, 1989). Synthesis of LTB₄ from endogenous AA results from the activation of two calcium-dependent events. First, the release of AA from membrane phospholipids and second, the activation of the 5-LO (Borgeat & Samuelsson, 1979; Borgeat *et al.*, 1983; Rouzer & Kargman, 1988). In the latter instance, 5-LO activation is likely to involve a calcium-dependent translocation of the enzyme to the plasma membrane (Rouzer & Kargman, 1988). Thus, the onset of LT synthesis by stimuli such as those listed above may well be related to their

calcium-mobilizing properties.

The stimulatory characteristics of LTB₄ towards neutrophils resemble those of other receptor-dependent soluble neutrophil agonists, i.e. fMLP, PAF and C5a (Naccache *et al.*, 1979; Clancy *et al.*, 1983; Naccache *et al.*, 1986). These compounds bind to specific cell-surface receptors to stimulate the phospholipase C-dependent release of diacylglycerol and inositol trisphosphate, a process mediated by pertussis toxin-sensitive, guanine nucleotide-binding proteins (G proteins) (Becker *et al.*, 1985; Naccache *et al.*, 1986; Shirato *et al.*, 1988). It is through this sequence of events that soluble agonists elicit several neutrophil functional responses, such as degranulation, superoxide anion generation, adherence, locomotion, and activation of the 5-LO (McDonald *et al.*, 1991). LTB₄ elicits similar responses, but in contrast to PAF, fMLP and C5a, it has not yet been shown to stimulate 5-LO activity.

Nevertheless, such a possibility was raised in recent reports from our laboratory, in which we investigated the mechanism whereby exogenous AA activates polymorphonuclear leukocytes (PMNL) (McColl *et al.*, 1989; Naccache *et al.*, 1989b). In these previous studies, we showed that LTB₄ synthesis and/or triggering of LTB₄ receptors significantly accounted for the ability of exogenous AA to stimulate an increase in intracellular calcium mobilization and activation of the 5-LO. Exogenous AA induced a dose-dependent mobilization of calcium that was inhibited by pertussis toxin, or upon pre-incubation of neutrophils with LTB₄; similarly, exogenous AA-induced 5-LO product synthesis was inhibited by pertussis toxin. These observations prompted us to investigate potential stimulatory effects of LTB₄ itself on the 5-LO metabolic pathway in neutrophils, as such effects of LTB₄ could represent an important feedback amplification mechanism in the production of this potent pro-inflammatory mediator.

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Methods

Cell separation

Blood was collected by venepuncture with heparin used as an anticoagulant, and following dextran sedimentation of erythrocytes, PMNL were purified by centrifugation on Ficoll-Plaque cushions (Pharmacia, Dorval, Québec, Canada). The erythrocytes remaining in the final pellet were removed by hypotonic lysis with water (30 s), and the cells were resuspended in HBSS buffered with HEPES (10 mM, pH 7.4) and supplemented with calcium (1.6 mM) and magnesium (1 mM), at a final concentration of 5×10^6 PMNL ml⁻¹ (unless otherwise stated). The entire cell separation was carried out at room temperature. The percentage of PMNL in the cell suspensions used in this study exceeded 96%, and cell viability was greater than 98%, as determined by trypan blue exclusion. The platelet/leukocyte ratio varied from 2 to 5.

Cell incubations

Cell suspensions were warmed to 37°C for 5 min before incubation with substrate and/or stimulus. All agonists, including exogenous AA and D₂-LTB₄, were dissolved in dimethyl sulphoxide (DMSO) and added to 1.0 ml aliquots of the cell suspension; the final concentration of DMSO (maximum of 0.3%) consistently failed to stimulate any detectable leukotriene synthesis. In the case of 15-HpETE, a stock solution (3 mM in ethanol) was diluted 10 fold in 0.6 mM sodium carbonate (to form the sodium salt of the fatty acid), and 10 µl of the resulting solution was added to 1.0 ml aliquots of the cell suspensions, to yield a final concentration of 3 µM. After the desired incubation time with the stimuli, the cells were denatured by the addition of 1.0 ml of an ice-cold mixture of methanol:acetonitrile (50:50 v:v) containing internal standards: 12.5 ng·ml⁻¹ of prostaglandin B₂ and 19-OH prostaglandin B₂. The samples were then stored at -20°C before reversed-phase h.p.l.c. (r.p.-h.p.l.c.) analysis.

In the experiments involving pertussis toxin, the cell suspensions were pre-incubated with the toxin at a final concentration of 0.5 µg ml⁻¹ for 3 h at 37°C. During this time, the cells were gently swirled in a rotary water bath (New Brunswick Scientific, Edison, New Jersey, U.S.A.); calcium and magnesium were added 20 min before stimulation. In the experiments involving MK-886 or LY-223982, the drugs were added to the cells 1 min before stimulation; both compounds were dissolved in DMSO.

Analysis of lipoxigenase products by r.p.-h.p.l.c.

Analysis of lipoxigenase products was performed by r.p.-h.p.l.c. as described previously, using an on-line extraction procedure (Borgeat *et al.*, 1990). Briefly, the denatured cell suspensions were centrifuged at 600 g (4°C, 10 min) to remove precipitated material, and the supernatants were directly injected onto a Radial Pak Resolve C₁₈ cartridge (5 × 100 mm, 5 µm particles, Waters Millipore, Milford, Massachusetts, U.S.A.) protected by Guard-Pak Resolve C₁₈ and silica cartridges (Waters Millipore). The various lipoxigenase products were eluted at 1.5 ml min⁻¹ using gradients of organic solvents and a change of pH. Elution was monitored using fixed-wavelength ultra-violet (u.v.) photometers at 229 and 280 nm; quantification was performed by comparing peak heights of each compound with those of calibrated standards, after correction for recovery using the internal standard, prostaglandin B₂. The lower limit of detection was 0.5 ng at 280 nm and 1 ng at 229 nm. In the experiments involving D₂-LTB₄, h.p.l.c. analysis of the samples was performed as described above, except that acetic acid was used instead of phosphoric acid to acidify the

various mobile phases, and LTB₄-containing h.p.l.c. eluate fractions were collected for GC-MS analysis.

GC-MS analysis of LTB₄

The eluate fractions containing LTB₄ were evaporated under a stream of nitrogen at 40°C. The trimethylsilyl ether methyl ester derivative of LTB₄ was obtained by successive treatment with ethereal diazomethane in methanol (5 min at 20°C), and with N-methyl-N-trimethylsilyltrifluoroacetamide and dimethylformamide (25 µl each) for 60 min at 50°C. The reagents were evaporated under a stream of nitrogen and the samples were dissolved in hexane prior to injection. GC-MS analysis (multiple ion monitoring) was performed on a VG MicroMass MM-16 (Altrincham, Cheshire) mass spectrometer coupled to a Varian 3700 GC, using a fused silica capillary column (Durabond DB-1, 0.25 mm I.D. × 15 m, J&W Scientific Inc., Folsom, California, U.S.A.) directly connected to the source. Samples were injected by an on-column injection technique and He was used as carrier gas (81 kPa). The injection temperature was 140°C, and after 2 min, the oven temperature was increased to 260°C at the rate of 10°C min⁻¹. The D₂-LTB₄ and D₀-LTB₄ derivatives co-eluted at 14.47 min. Analysis was performed in the electron impact mode. Ionizing energy was 25.0 eV, the trap current 200 µA and the source was maintained at 250°C. Ion current was recorded at m/z 463.3 and 465.3 (M-31); these ions represented 4% of the base peak (m/z 129) in both spectra. LTB₄ was quantified from a calibration curve generated from mixtures of LTB₄ (0–20 ng) and D₂-LTB₄ (12 ng) and the contribution of LTB₄ to D₂-LTB₄ at m/z 465.3 was subtracted.

Materials

PAF, fMLP, HEPES, pertussis toxin and arachidonic acid were obtained from the Sigma Chemical Company (St-Louis, Missouri, U.S.A.). Hank's Balanced Salt Solution (HBSS) was from GIBCO (Burlington, Ontario, Canada), and all solvents were high performance liquid chromatography (h.p.l.c.) grade from Anachemia (Montréal, Québec, Canada). As previously described for 13-hydroperoxy-octadecadienoic acid (Gardner, 1975), soybean lipoxigenase was used to synthesize 15-HpETE from AA. AA was purified on a silicic acid column before use for enzymatic synthesis of 15-HpETE, which was then purified by straight-phase h.p.l.c. It must be stressed that it is critical to purify AA and 15-HpETE freshly before use, because of the natural reactivity of these compounds. Leukotriene B₄, D₂-LTB₄ (14,15-dideuterio) and MK-886 were generous gifts from Dr Robert N. Young of the Merck-Frosst Centre for Therapeutic Research (Pointe-Claire, Québec, Canada).

Statistical analyses

Where mentioned, statistical significance was assessed by Student's paired *t* test.

Results

Conversion of exogenous AA into LTB₄ stimulated by D₂-LTB₄

To determine whether LTB₄ has the ability to stimulate its own synthesis from endogenous or exogenous substrate, PMNL (2×10^6 ml⁻¹) were incubated with 30 nM D₂-LTB₄ in the presence or absence of 3.3 µM exogenous AA for 2.5 min. Use of D₂-LTB₄ as a stimulus allows the quantification by GC-MS procedures of LTB₄ generated from AA. Figure 1 shows that PMNL exposed to 30 nM D₂-LTB₄ failed to synthesize LTB₄ in amounts significantly above control,

Figure 1 Stimulation by 14,15-dideuterio-leukotriene B₄ (D₂-LTB₄) of LTB₄ synthesis from exogenous arachidonic acid (AA). PMNL were incubated at 37°C for 2.5 min under the conditions indicated in Methods; samples were processed by r.p.-h.p.l.c. and LTB₄-containing fractions were collected and further analysed by GC-MS. The results are expressed as the mean $\pm$ s.e. mean of averaged triplicate incubations from 3 independent experiments. NS: not statistically significant; ** P < 0.011.

indicating that D₂-LTB₄ did not stimulate the formation of LTB₄ from endogenous AA. Consistent with these observations, no 5-HETE (5(S)-hydroxy-6,8,11,14-eicosatetraenoic acid) could be detected under these conditions. Similar results

were obtained with 100 nM D₂-LTB₄ (not shown). Incubation of PMNL with 3.3 μM AA did not elicit a significant synthesis of LTB₄. In contrast, co-addition of 3.3 μM AA and 30 nM D₂-LTB₄ consistently resulted in a marked increase in the synthesis of LTB₄. Under such conditions, small amounts of 5-HETE were sometimes detected. These results indicate a stimulatory effect of LTB₄ on 5-LO activity in PMNL.

Stimulation by LTB₄ of 5-LO-dependent conversion of 15-HpETE into 5,15-DiHETE

The stimulatory effect of LTB₄ on 5-LO activity was confirmed and further characterized with an alternative substrate, 15-HpETE. The resulting product, 5,15-DiHETE, can be measured by r.p.-h.p.l.c. and u.v. detection at 229 nm. PMNL were incubated with 3.0 μM 15-HpETE in presence or absence of 100 nM LTB₄ for 15 min, the reactions were stopped, and the supernatants analysed by r.p.-h.p.l.c. When both LTB₄ and 15-HpETE were added to PMNL, 2 major absorbance peaks were detected at 229 nm (Figure 2a): 15-hydroxy eicosatetraenoic acid (15-HETE), the reduction product of 15-HpETE, and 5,15-DiHETE, the reduced 5-LO metabolite of 15-HpETE. In contrast, when 15-HpETE only was added, a much smaller peak of 5,15-DiHETE was detected, along with the 15-HETE peak (Figure 2b). Finally, no 5,15-DiHETE could be detected in cells stimulated with LTB₄ alone (Figure 2c). Therefore, as demonstrated by this experiment, LTB₄ stimulated the 5-LO-mediated transformation of 15-HpETE into 5,15-DiHETE, thus confirming our previous observations using AA as exogenous substrate.

To determine the time course of the 5-LO transformation of 15-HpETE into 5,15-DiHETE, PMNL were incubated for up to 15 min with 3.0 μM 15-HpETE, either alone or with 100 nM LTB₄. When PMNL were incubated with 15-HpETE

Figure 2 Stimulation by leukotriene B₄ (LTB₄) of the transformation of exogenous 15-HpETE by the 5-lipoxygenase (5-LO). PMNL were incubated at 37°C for 15 min under the following conditions: (a) 100 nM LTB₄ and 3.0 μM 15-HpETE; (b) 3.0 μM 15-HpETE alone; (c) 100 nM LTB₄ alone. This figure shows r.p.-h.p.l.c. chromatograms (upper tracings, u.v. absorbance at 229 nm with attenuation set at 0.20 a.u.f.s.; lower tracings, u.v. absorbance at 280 nm with attenuation set at 0.05 a.u.f.s.) of lipoxygenase products in the denatured incubation media.

alone, a small increase in 5,15-DiHETE synthesis occurred, which plateaued at about 3 min (Figure 3a). Co-addition of 100 nM LTB₄ led to a greatly enhanced synthesis of 5,15-DiHETE, relative to unstimulated cells, with a similar time course. The effect of increasing concentrations of LTB₄ on the 5-LO metabolism of 15-HpETE is shown in Figure 3b. Activation of the 5-LO was detected with concentrations of LTB₄ lower than 1 nM, and was nearly maximal at 1 μ M LTB₄. LTB₄ alone neither stimulated the synthesis of 5,15-DiHETE, nor that of endogenous AA-derived 5-LO products (as determined by r.p.-h.p.l.c. and u.v. detection), regardless of the concentration used (not shown).

Inhibition by MK-886 of the LTB₄-induced activation of 5-LO

MK-886, an indole derivative, has recently been described as a specific LT synthesis inhibitor that blocks the A23187- or fMLP-induced 5-LO translocation from the cytosol to the plasma membrane (Kargman *et al.*, 1991). We therefore conducted experiments to determine whether pre-incubation of PMNL with this drug would result in the inhibition of the 5-LO activation induced by LTB₄. PMNL were pre-treated with increasing concentrations of MK-886 (up to 300 nM), and incubated for 15 min with 3.0 μ M 15-HpETE, either

alone or with 100 nM LTB₄. Figure 4 shows that MK-886 dose-dependently inhibited the LTB₄-induced 5,15-DiHETE formation, a maximal effect being reached between 100 and 300 nM MK-886. The maximal inhibition averaged $68.0 \pm 3.1\%$ (mean $\pm$ s.e.mean) in 5 independent experiments.

Structural specificity of the LTB₄-induced activation of 5-LO

We next investigated whether the LTB₄-induced activation of the 5-LO was specific, using the ω -oxidation metabolite of LTB₄, 20-hydroxy-LTB₄, and a stereoisomer of LTB₄, Δ^6 -trans-12-epi-LTB₄. PMNL were incubated for 15 min with 3.0 μ M 15-HpETE, either alone or with increasing concentrations of LTB₄, Δ^6 -trans-12-epi-LTB₄, or 20-hydroxy-LTB₄ (Figure 5). All compounds investigated failed to stimulate the formation of 5-LO products when added by themselves, regardless of their respective concentrations (not shown). In contrast, co-incubation with 15-HpETE induced a concentration-dependent activation of the 5-LO by all compounds. However, LTB₄ was approximately 100 times more active than 20-hydroxy-LTB₄, and about 300 times more active than Δ^6 -trans-12-epi-LTB₄. These results indicate that the activation of the 5-LO by LTB₄ shows a high degree of structural specificity (including stereospecificity).

Effect of pertussis toxin on the LTB₄-induced activation of 5-lipoxygenase

Pertussis toxin-sensitive G proteins are involved in the trans-membrane signalling process whereby LTB₄ exerts its various effects on neutrophil functions. We therefore examined whether the LTB₄-induced activation of the 5-LO was also G protein-dependent. PMNL suspensions were pre-incubated with either pertussis toxin or diluent, and then exposed to 3.0 μ M 15-HpETE, alone or with 100 nM LTB₄ for 15 min. Pre-incubation with pertussis toxin strongly inhibited ($69.6 \pm 10.7\%$, $n = 7$, $P < 0.001$) the LTB₄-induced 5,15-DiHETE formation from 15-HpETE, relative to control preparations. Finally, the minor 5,15-DiHETE synthesis that occurs when

Figure 3 Time course and concentration dependency of the leukotriene B₄ (LTB₄)-induced 5-lipoxygenase (5-LO) transformation of 15-HpETE into 5,15-DiHETE. PMNL were incubated (a) for varying lengths of time at 37°C with 3.0 μ M 15-HpETE, by itself or with 100 nM LTB₄; (b) for 15 min at 37°C with increasing concentrations of LTB₄, either by itself (not shown) or with 3.0 μ M 15-HpETE. Note that in (b), the 5,15-DiHETE levels (17.5 ± 0.9 ng per 5×10^6 PMNL) resulting from exposure to 15-HpETE alone have been subtracted, so as to depict only the effect of LTB₄. Symbols: (□) 15-HpETE alone; (■) 15-HpETE and LTB₄. These experiments are representative of three (a) and eight (b); each data point is the mean with s.e.mean (vertical bars) of triplicate incubations.

Figure 4 Inhibition of the leukotriene B₄ (LTB₄)-induced 5-lipoxygenase (5-LO) activation by MK-886. PMNL were pretreated with increasing concentrations of MK-886 (for 1 min at 37°C) and incubated for 15 min with 3.0 μ M 15-HpETE, alone or with 100 nM LTB₄. The 5,15-DiHETE levels resulting from exposure to 15-HpETE ($\pm$ MK-886, in the absence of LTB₄) have been subtracted, so as to only depict the effect of MK-886 on the 5-LO activation induced by LTB₄. The results are expressed as the mean $\pm$ s.e.mean (vertical bars) of averaged triplicate incubations from five independent experiments. * $P < 0.036$.

Figure 5 Structural specificity of the leukotriene B_4 (LTB_4)-induced activation of the neutrophil 5-lipoxygenase (5-LO). PMNL were incubated for 15 min at 37°C with 3.0 μ M 15-HpETE, alone or with increasing concentrations of LTB_4 (■), Δ^6 -*trans*-12-*epi*- LTB_4 (Δ) or 20-hydroxy- LTB_4 (▲). The 5,15-DiHETE levels (9.0 ± 0.2 ng per 5×10^6 PMNL) resulting from exposure to 15-HpETE alone have been subtracted, so as to depict only the effect of the compounds investigated. This experiment is representative of four; each data point is the mean $\pm$ s.e.mean (vertical bars) of triplicate incubations.

PMNL are incubated with 15-HpETE alone was not significantly affected by pretreatment of the cells with pertussis toxin in 7 independent experiments ($P = 0.24$; data not shown).

Inhibition of the LTB_4 -induced 5-LO activation by the LTB_4 receptor antagonist LY-223982

That the LTB_4 -induced 5-LO activation is stereospecific and probably involves a pertussis toxin-sensitive G protein led us to investigate further this process at the level of the LTB_4 receptor. PMNL were pre-incubated with 1.0 μ M of the LTB_4 receptor antagonist LY-223982 for 1 min and then exposed to 3.0 μ M 15-HpETE alone, or together with increasing concentrations of LTB_4 for 15 min. LY-223982 pretreatment induced a pronounced shift to the right of the dose-response curve for the LTB_4 -induced activation of the 5-LO (Figure 6). LY-223982 had no significant effect on the 5,15-DiHETE synthesis resulting from exposure of the cells to 15-HpETE alone in 5 independent experiments ($P = 0.32$; data not shown).

Discussion

It was previously observed that soluble neutrophil agonists which share many common actions with LTB_4 (namely, PAF, fMLP, C5a and IL-8) stimulate the 5-LO-dependent conversion of exogenous AA into leukotrienes in human PMNL (Lin *et al.*, 1982; Clancy *et al.*, 1983; Schröder, 1989). In a recent study, we further characterized the stimulatory effect of these neutrophil agonists on 5-LO activation through the use of an alternative substrate, 15-HpETE (McDonald *et al.*, 1991). We now show that, like the other soluble neutrophil agonists, LTB_4 activates the 5-LO in human PMNL.

First, we demonstrated that LTB_4 activates the human neutrophil 5-LO, but by itself does not appear to induce the formation of 5-LO products derived from endogenous substrate. This conclusion is based upon the observations that LTB_4 greatly increased the 5-LO-dependent metabolism of low micromolar concentrations of exogenous AA (Figure 1) and 15-HpETE (Figure 2), while it failed to induce a detec-

Figure 6 Inhibition by the receptor antagonist LY-223982 of the leukotriene B_4 (LTB_4)-induced 5-lipoxygenase (5-LO) activation. PMNL were incubated at 37°C for 15 min with 3.0 μ M 15-HpETE and increasing concentrations of LTB_4 , with (□) or without (■) prior treatment with 1.0 μ M LY-223982 (1 min at 37°C). The 5,15-DiHETE levels (2.0 ± 0.2 ng per 5×10^6 PMNL) resulting from exposure to 15-HpETE alone ($\pm$ LY-223982) have been subtracted, so as to depict only the effect of LY-223982 on 5-LO activation induced by LTB_4 . This experiment is representative of three; each data point is the mean $\pm$ s.e.mean (vertical bars) of triplicate incubations.

table synthesis of 5-LO products when added to the cells by itself. Although it is still possible that LTB_4 stimulated the release of endogenous AA and subsequent synthesis of LTB_4 in amounts too small to be detected by r.p.-h.p.l.c. or by our GC-MS assay (detection limit, 100 pg), our present results are in accordance with those of Prescott *et al.* (1984) who demonstrated that LTB_4 did not cause the release of [3 H]-AA from pre-labelled neutrophils. Similarly, Haurand & Flohé (1989) showed that LTB_4 failed to induce the formation of 5-LO metabolites from endogenous AA. Neither study however, investigated the transformation of exogenous substrate as in the present paper.

Further characterization of the stimulation by LTB_4 of the 5-LO-mediated metabolism of exogenous 15-HpETE revealed that the activation of the 5-LO by LTB_4 is a time- and concentration-dependent process (Figure 3). It must be emphasized here that the use of 3 μ M 15-HpETE entails an underestimation of 5-LO activation, as this substrate concentration is suboptimal (McDonald *et al.*, 1991). Our results also demonstrated that the LTB_4 -elicited 5-LO activation can be concentration-dependently inhibited by MK-886 (Figure 4). MK-886 is known to bind to the 5-LO-activating protein or FLAP (Dixon *et al.*, 1990), and has been shown to block the A23187- and fMLP-elicited translocation of the 5-LO to the plasma membrane, as well as the concomitant LT synthesis (Kargman *et al.*, 1991). Since MK-886 shows no effect on the 5-LO itself, its inhibitory effect on the LTB_4 -induced 5-LO activation suggests that the mechanism whereby LTB_4 activates the 5-LO probably involves translocation of the enzyme to the plasma membrane. However, whilst our experiments with MK-886 provide plausible evidence for such a mechanism, a formal proof would require the use of more direct approaches, such as immunoblotting or ELISA determination of 5-LO levels in subcellular fractions.

In addition, our data indicate that the activation of the 5-LO by LTB_4 results from the binding of LTB_4 to specific cell-surface receptors, and that the transmembrane signal thus generated is transduced by pertussis toxin-sensitive G

proteins. This assertion is supported by the results of several experiments. First, the ability of LTB₄ to stimulate 5-LO activation was strongly inhibited by pertussis toxin, a characteristic previously reported for other receptor-dependent actions of LTB₄ in neutrophils (Molski *et al.*, 1984; Becker *et al.*, 1985). It is therefore likely that the G protein(s) involved in the activation of the 5-LO by LTB₄ is the same as that implicated in the other actions of LTB₄. Second, our finding that the 5-LO activation induced by LTB₄ is stereospecific (Figure 5) is also in good agreement with the reported effects of LTB₄ on other neutrophil responses (Naccache *et al.*, 1989a). Finally, the involvement of the LTB₄ cell-surface receptor was confirmed through the use of the LTB₄ receptor antagonist LY-223982 (Jackson *et al.*, 1988), which effectively inhibited the LTB₄-induced 5-LO activation (Figure 6).

The preceding findings suggest that the presence of LTB₄ at inflammatory foci could potentially stimulate the synthesis of LTB₄ itself, provided that exogenous AA is available to the activated cells. That these conditions are fulfilled in a number of pathophysiological conditions makes such a scenario realistic. Indeed, LTB₄ is known to be actively synthesized upon exposure of phagocytes to soluble or particulate agonists, and has been detected in many inflammatory conditions in man (Ford-Hutchinson, 1990). Similarly, micromolar concentrations of AA have been measured in inflammatory lesions (Barr *et al.*, 1984; Greaves,

1986). Moreover, AA could be supplied to activated neutrophils through cellular interactions; in this respect, it was recently reported that PMNL stimulated by soluble agonists can effectively synthesize LTB₄ using AA derived from activated platelets (Fiore & Serhan, 1990; Palmantier & Borgeat, 1991). Therefore, the generation of LTB₄ at inflammatory sites, as well as its ability to synergise with AA to produce positive feedback on its own synthesis, could represent a powerful amplification mechanism of the inflammatory reaction *in vivo*, especially when considering the potent action of the lipid mediator on phagocyte recruitment. In a similar fashion, the presence of PAF, C5a, IL-8 and formylated peptides at inflammatory foci may also enhance ongoing leukotriene synthesis by PMNL, since these compounds share with LTB₄ the ability to activate the 5-LO (Schröder, 1989; McDonald *et al.*, 1991). Studies are in progress to assess potential autocrine effects of LTB₄ on leukotriene synthesis and on other functional responses in activated PMNL undergoing leukotriene synthesis.

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