Leukotriene B₄ Strongly Increases Monocyte Chemoattractant Protein-1 in Human Monocytes

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**Objective**—Leukotriene B₄ (LTB₄), a product of the 5-lipoxygenase (5-LO) pathway of arachidonic acid metabolism, has been implicated in atherosclerosis. However, the molecular mechanisms for the atherogenic effect of LTB₄ are not well understood. This study is to determine candidate mechanisms.

**Method and Results**—Primary human monocytes were treated with LTB₄ and the supernatant was analyzed for cytokine/chemokine production by an immuno-protein array. This analysis revealed a strong increase of the monocyte chemoattractant protein-1 (MCP-1), a proinflammatory cytokine. Follow-up analyses with MCP-1 enzyme-linked immunosorbent assay (for quantitation of MCP-1 protein) and real-time polymerase chain reaction (PCR) (for MCP-1 mRNA) demonstrated that LTB₄ strongly induced expression of MCP-1 protein and mRNA in a time-dependent and dose-dependent fashion. This induction was effectively abolished by CP-105,696, an antagonist for the LTB₄ receptor BLT₁. Selective inhibitors of ERK1/2 or JNK MAPK effectively blocked the LTB₄-induced MCP-1 production. Furthermore, LTB₄ increased NF-κB DNA binding activity, which was blocked by CP-105,696.

**Conclusions**—LTB₄ strongly induces MCP-1 production in primary human monocytes. This induction is mediated through the BLT₁ pathway increasing MCP-1 transcription. Activation of ERK1/2 or JNK MAPK is essential for this induction. The NF-κB activation may be involved in LTB₄-induced MCP-1 expression. The LTB₄-induced MCP-1 in human monocytes may play a critical role in the atherogenicity of LTB₄. (Arterioscler Thromb Vasc Biol. 2004;24:1783-1788.)

**Key Words:** leukotriene B₄ ■ monocyte chemoattractant protein-1 ■ atherosclerosis ■ human monocytes

Leukotriene B₄ (LTB₄), a product of the 5-lipoxygenase (5-LO) pathway of arachidonic acid metabolism, is a potent chemoattractant and proinflammatory mediator involved in the pathogenesis of several inflammatory diseases including atherosclerosis.¹⁻³ The biological effects of LTB₄ are mediated by activation of 2 G-protein coupled receptors, the high-affinity receptor BLT₁ and the low-affinity receptor BLT₂.⁴⁻⁵ Studies with BLT₁-specific and BLT₂-specific antagonists demonstrate that BLT₁ activation is involved in inflammation and immunologic effects of LTB₄, whereas the function of BLT₂ is not yet well defined.⁶

Recent studies suggest a strong link between LTB₄ pathways with atherosclerosis. For example, human atherogenic plaques produce LTB₄,⁷ and expression of both BLT₁ and BLT₂ increases with the progression of atherosclerotic lesions.⁸ Treatment of atherosclerosis-prone mice (apolipoprotein E [ApoE] or low-density lipoprotein receptor [LDLR]-deficient mice) with the BLT₁-specific antagonist CP-105,696⁹,¹⁰ markedly decreased lesion size.¹¹ Interestingly, the anti-atherogenic effects of CP-105,696 were diminished in mice deficient in the chemoattractant monocyte chemattractant protein-1 (MCP-1),¹¹ indicating a critical role for MCP-1 in mediating the LTB₄ atherogenic signals.

MCP-1 is a prototype of the C-C chemokine β subfamily and exhibits the most potent chemotactic activity for monocytes.¹² Overexpression of MCP-1 contributes to the development of atherosclerosis in mouse models.¹³ Deficiency of either MCP-1 or its cognate high-affinity receptor C-C chemokine receptor 2 (CCR2) results in a marked decrease in atheromas and fewer monocytes in vascular lesions.¹⁴,¹⁵ Additionally, therapeutic gene transfer of a dominant-negative MCP-1 mutant attenuated the development of early atherosclerosis and also limited progression of preexisting atherosclerotic lesions in ApoE-null mice.¹⁶

Despite the critical role played by LTB₄ in atherogenesis, the molecular mechanisms for these activities are poorly understood. In this study, we investigated specifically whether LTB₄ regulates MCP-1 production in primary human monocytes to broaden the mechanistic understanding for LTB₄-induced atherosclerosis. Our study shows that LTB₄ induced MCP-1 protein by several hundred-fold in primary human monocytes. LTB₄ induced MCP-1 mRNA by 500-fold
to 600-fold, suggesting that LTB₄-induced MCP-1 protein expression was accomplished by a transcriptional mechanism. The BLT₁-specific antagonist CP-105,696 effectively blocked this induction, indicating that this event is mediated through the BLT₁ signaling pathway. We further demonstrated that inhibitors of ERK1/2 or JNK MAPK abolished this induction. Additionally, we showed that LTB₄ increased NF-κB DNA binding activity, which was blocked by CP-105,696. Taken together, these results suggest that the pathway for LTB₄-induced MCP-1 production involves activation of ERK1/2 or JNK and activation of NF-κB. The LTB₄ induced MCP-1 expression in primary monocytes may play a pivotal role in the atherogenicity of LTB₄.

Methods

Materials
The following reagents were obtained from Invitrogen: DMEM and RPMI-1640 medium, fetal bovine serum, and fetal calf serum, and TRIZOL reagents. The RayBio™ Human Cytokine Array V was obtained from RayBiotech, Inc. The enriched human monocytes were purchased from Biological Specialty Inc. The Calcium^{2+} Assay Kit was obtained from Molecular Devices Corporation. The leukotrienes were from BIOMOL. CP-105,696 can be made according to the procedure of Koch et al.17 The human MCP-1 Quantikine kit was purchased from R&D Systems, Inc. SB202190 was purchased from Calbiochem, and PD98059 was from Cell Signaling. U0126 and Curcumin were from Sigma. TaqMan reagents for cDNA synthesis and real-time polymerase chain reaction (PCR) were purchased from Applied Biosystems. Human BLT₁ cDNA was a gift from Dr. Takao Shimizu at University of Tokyo.

Isolation and Treatment of Primary Human Monocytes
Ten milliliters of lymphocyte separation medium (LSM/Ficoll) was slowly added to 40 mL of the enriched human monocytes from Biological Specialty Inc. and the mixture was spun at 600g for 20 minutes. The peripheral blood mononuclear cell layer was collected and filled with PBS to 50 mL, and then spun at 1000 rpm for 5 minutes. The cell pellet was resuspended in 5 mL RPMI-1640 medium containing 10% fetal calf serum. Sheep red blood cells were added to the pellet and incubated for 15 minutes at 37°C followed by centrifugation at 1000 rpm for 5 minutes. The pellet was kept at −20°C for 6 minutes, and then was gently resuspended in 25 mL of phosphate-buffered saline. The cells were further purified with 10 mL LSM/Ficoll by repeating the same steps as described. The mononuclear layer at the interface was collected. The red blood cells were lysed if necessary. Monocytes were resuspended in 50 mL of RPMI-1640 medium containing 10% fetal calf serum.

The primary monocytes in RPMI-1640 medium were pelleted and resuspended in Hank’s balanced salt solution (HBSS) buffer (with calcium and magnesium) containing 2% Pen/Strep and 1% heat-inactivated fetal bovine serum. The cells were seeded in 24-well plates at a density of 2×10⁶ cells per well and incubated with various concentrations of LTB₄, LTC₄, LTD₄, LTE₄ or carboxyl methyl platelet-activating factor (c-PAF). For inhibitor studies, monocytes were pretreated for 30 minutes with CP-105,696 (LTB₄ receptor antagonist), PD98059 (ERK1/2 MAPK inhibitor), U0126 (ERK1/2 MAPK inhibitor, 10 μmol/L), curcumin (JNK MAPK inhibitor, 10 μmol/L), and SB202190 (p38 MAPK inhibitor, 1 μmol/L) before the addition of 30 nM LTB₄. Unless specified, cells were treated overnight before the medium was collected and centrifuged to remove any residual cells. The cell-free supernatant was stored at −80°C for MCP-1 determination, and cells were dissolved in TRIZOL reagent for total RNA isolation.

Analysis of Human Cytokine Array and MCP-1 Determination
The human cytokine array studies were conducted using the RayBio™ Human Cytokine Array V kit following the manufacturer’s instructions. Primary human monocytes were treated overnight with ethanol or 60 nM LTB₄, and the cell-free supernatant was collected. One milliliter of each sample was used for analysis. The human MCP-1 concentrations were determined by the Quantikine kit according to the manufacturer’s instructions.

The Fluorometric Imaging Plate Reader Assay
The fluorometric imaging plate reader (FLIPR) assay was used to confirm the BLT₁ antagonist activity of CP-105,696. HEK 293T cells stably overexpressing BLT₁ (Zhao et al, unpublished data) were seeded at a density of 22000 cells/well in 384-well plates 24 hours before the assay. The FLIPR assays were performed using the Calcium^{2+} Assay Kit from Molecular Devices Corporation according to the manufacturer’s instructions. Briefly, 10 μL of HBSS with 20 mmol/L HEPES were added to Component A from the kit to make a 10× loading dye. Then probedinced (Sigma P-8761) was freshly added to the 10× loading dye. To each well, 25 μL of the 1× loading dye was added and incubated with cells for 1 hour at 37°C. CP-105,696 was diluted with 1× HBSS/HEPES/probe- cific buffer from which 25 μL was added 5 minutes before the addition of 25 μL of LTB₄ at a final concentration of 5 nM. Plates were then read for 5 more minutes. Maximal peak values were used for calculation.

Electrophoretic Mobility-Shift Assay
NF-κB DNA binding activity was determined using Gel Shift Assay Systems kits from Promega following manufacturer’s instructions. Briefly the nuclear extract was isolated from primary human monocytes treated with 30 nM LTB₄, 30 nM LTC₄, or 30 nM LTB₄ plus 3 nM CP-105,696 overnight. Nuclear proteins (6 μg) were added to the 1× binding buffer provided with the kit in the presence or absence of a 100-fold excess of the unlabeled NF-κB oligonucleotide (specific competitor) or the AP-1 oligonucleotide (nonspecific competitor) and incubated at room temperature for 10 minutes. The 32P-labeled probe (5’-AGT TGA GGG GAC TTT CCC AGG C-3’) was then added to the reaction and incubated at room temperature for 20 minutes. The protein–DNA complex was resolved on a 4% acrylamide gel, which was then dried and exposed for autoradiography.

RNA Isolation and Real-Time Quantitative PCR
Total RNA was extracted from the treated primary human monocytes using the TRIZOL reagent according to the manufacturer’s instructions. Reverse-transcription reactions and TaqMan PCRs were performed according to the manufacturer’s instructions (Applied Biosystems). Sequence-specific amplification was detected with an increased fluorescent signal of FAM (reporter dye) during the amplification cycles. Amplification of human 18S RNA was used in the same reaction of all samples as an internal control. Gene-specific mRNA was subsequently normalized to 18S RNA. Levels of human MCP-1 mRNA were expressed as fold difference of compound-treated cells against vehicle-treated cells.

Oligonucleotide/primer/human MCP-1 were designed using the Primer Express program and were synthesized by Applied Biosystems. These sequences (5’ to 3’) are as follows: forward primer (CAACCGAAGTGTTGTCAGGAT), 3’ (6FAM-CATGGACCACCTGGACAAGGCAAACC-TAMRA), and reverse primer (AGTGAAGGTGTCAGTCTGGAAGT).

Results
LTB₄ Strongly Increases MCP-1 Production in Primary Human Monocytes
To determine whether LTB₄ can regulate expression of proinflammatory cytokines/chemokines, primary human
monocytes were treated with 60 nM LTB₄ overnight, and cell-free supernatant was collected and hybridized with an immunoblot array that contains 79 human cytokines/chemokines. Compared with the vehicle-treated control (Figure 1A), LTB₄ induced expression of MCP-1 protein by several hundred-fold (Figure 1B). In addition, LTB₄ treatment also resulted in modest changes of other cytokines/chemokines including increases in placental growth factor and transforming growth factor (TGF)-β3 (TGF-B3) and a decrease in macrophage inflammatory protein-1β (MIP-1β) (Figure 1B). This study solely focused on the investigation of LTB₄-induced MCP-1 expression.

Follow-up experiments were conducted to confirm the observation of LTB₄-induced MCP-1 production. In these experiments, primary monocytes were treated overnight with vehicle (ethanol) or 30 nM LTB₄ for 1, 4, 16, 24, and 36 hours to define the time kinetics for LTB₄-induced MCP-1 production. LTB₄ increased MCP-1 protein in a time-dependent fashion, and significant increase was observed from 16 to 36 hours (Figure 2B). In the vehicle-treated cells, the basal level of MCP-1 was also slightly increased with time (Figure 2B).

LTB₄ Induces MCP-1 Transcription

To investigate whether the increase of MCP-1 protein in supernatants resulted from an increased transcription of MCP-1, total RNA from treated primary human monocytes was extracted and the endogenous MCP-1 mRNA was determined by real-time PCR (TaqMan). Consistent with the observation that LTB₄ strongly increased MCP-1 protein, LTB₄ at 30 nM increased MCP-1 mRNA by >200-fold (Figure 3A). In contrast, the cysteinyl leukotrienes C₄, D₄, and E₄ did not significantly regulate MCP-1 expression (Figure 3A). Similar to the previous reports, c-PAF also increased MCP-1 mRNA by 50-fold (Figure 3C).

LTB₄ robustly increased MCP-1 protein in a dose-dependent manner with a half-maximum stimulation (EC₅₀) value between 3 and 10 nM (Figure 2A). Primary human monocytes were treated with vehicle (ethanol) or 30 nM LTB₄ for 1, 4, 16, 24, and 36 hours to determine the time kinetics for LTB₄-induced MCP-1 production. LTB₄ increased MCP-1 protein in a time-dependent fashion, and significant increase was observed from 16 to 36 hours (Figure 2B). In the vehicle-treated cells, the basal level of MCP-1 was also slightly increased with time (Figure 2B).

Figure 1. LTB₄ strongly induces MCP-1 protein in primary human monocytes. Primary human monocytes were isolated and treated overnight with vehicle (A) or 60 nM LTB₄ (B). The cell-free supernatant was collected and hybridized with the membrane blot of human cytokine array that contains 79 human cytokines/chemokines along with 6 positive (the 4 dots at the top left and 2 dots at the bottom right) and 2 negative controls (at bottom right next to the 2 positive controls). The spots for MCP-1, placental growth factor, TGF-B3, and MIP-1β are indicated by arrows. C, Primary human monocytes were treated with vehicle (EtOH) or 30 nM LTB₄, LTC₄, LTD₄, LTE₄, or 1 μmol/L c-PAF overnight, and the MCP-1 concentrations in supernatants were determined by an enzyme-linked immunosorbent assay. Each value represents the mean±SD of 4 determinations.

Figure 2. LTB₄ increases MCP-1 protein in a dose- and time-dependent fashion. Primary human monocytes were isolated and treated with various concentrations of LTB₄ overnight (A) or with 30 nM LTB₄ for various times (B). Cell-free supernatants were collected and the MCP-1 levels were determined by an enzyme-linked immunosorbent assay. Each value represents the mean±SD of 4 determinations.

Figure 3. LTB₄ increases MCP-1 transcription. A, Primary human monocytes were isolated and treated overnight with ethanol (EtOH) or 30 nM LTB₄, LTC₄, LTD₄, LTE₄, or 1 μmol/L c-PAF (A) or with various concentrations of LTB₄ (B). At the end of treatment, cells were harvested and total RNA was extracted, and TaqMan analysis was conducted for MCP-1 mRNA. Results are normalized as fold of control values (treated cells versus vehicle), and data are the mean±SD of 3 determinations.
CP-105,696 Effectively Blocks LTB₄-Induced MCP Production
CP-105,696 is a synthetic BLT₁ antagonist that potently and specifically blocks LTB₄-mediated functional responses.⁹,¹⁰

In our experiments, CP-105,696 effectively inhibited LTB₄-stimulated calcium flux in HEK 293T cells stably overexpressing BLT₁ (Figure 4A). To determine whether the LTB₄-induced MCP-1 expression is mediated by BLT₁, primary monocytes were treated with CP-105,696 in the presence and absence of 30 nM LTB₄. MCP-1 levels in the supernatant were determined by enzyme-linked immunosorbent assay. This increase was effectively blocked by CP-105,696 in a dose-dependent manner with an IC₅₀ value of 34 nM (Figure 4B). These data suggest that the induction of MCP-1 by LTB₄ is mediated through the BLT₁ pathway.

Inhibition of ERK1/2 and/or JNK MAPKs Abolished LTB₄-Induced MCP-1 Expression

It has been previously shown that activation of BLT₁ may result in activation of variety protein kinases.¹⁹,²⁰ We asked whether ERK1/2, JNK or p38 MAPKs are involved in the LTB₄-induced MCP-1 protein expression. Figure 5A shows that LTB₄-induced MCP-1 expression was effectively blocked by the ERK1/2 MAPK inhibitor PD98059 in a dose-dependent fashion, and that this induction was completely abolished by PD98059 at 5 μmol/L. U0126, another inhibitor of ERK1/2 MAPK, also completely blocked MCP-1 induction at 10 μmol/L (Figure 5B), indicating that activation of ERK1/2 is an essential step for LTB₄-induced MCP-1 expression. The JNK MAPK inhibitor curcumin (10 μmol/L) completely blocked this induction as well (Figure 5B), suggesting that activation of JNK is also necessary for the LTB₄-induced MCP-1 expression. In contrast, the p38 MAPK inhibitor SB202190 had no effect on MCP-1 expression (data not shown), suggesting that p38 activation is not required for this signaling pathway.

LTB₄ Increases NF-κB DNA Binding Activity in Primary Human Monocytes

It has been reported that NF-κB plays a key role in regulating MCP-1 transcription.²¹ To elucidate the molecular basis for LTB₄-induced MCP-1 expression, we determined whether LTB₄ could increase NF-κB DNA binding activity in primary human monocytes. Consistent with these results, nuclear extracts from LTB₄-treated but not LTC₄-treated cells showed a strong increase in NF-κB DNA binding activity (Figure 6A, lanes 2, 3, and 5). Furthermore, this activity was effectively abolished by the BLT₁ antagonist CP-105,696 (Figure 6A, lane 4). The specificity for LTB₄-induced NF-κB activity was demonstrated by competition experiments (Figure 6B). This NF-κB activity was completely abolished by a 100-fold molar excess of unlabeled NF-κB oligonucleotide (Figure 6B, lanes 3 and 4) but was not affected by the same molar excess of an unrelated oligonucleotide (Figure 6B, lanes 5 and 6). In addition to NF-κB, LTB₄ treatment did not significantly increase the DNA binding activity for AP-1, AP-2, CREB, and SP-1 (data not shown). Taken together, these results indicate that NF-κB was activated by LTB₄ and suggest that NF-κB may play an important role in LTB₄-induced MCP-1 expression in monocytes.

Discussion
Recent biologic and genetic findings implicate that the 5-LO pathway plays a critical role in atherosclerosis.¹⁸,²²–²⁴ Mehrabian et al reported that heterozygotes for the 5-LO gene on the LDLR⁻⁻ background had considerably reduced aortic lesions despite hypercholesterolemia as compared with the advanced lesions of LDLR⁻⁻ mice.²⁵ Dwyer et al showed that variant alleles of 5-LO genes were associated with a significant increase of carotid intima thickness.²⁶ Most recently, Helgadottir et al demonstrated a significant association between the gene encoding 5-LO activating protein (FLAP) and myocardial...

References

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