Hydroxyeicosanoids Bind to and Activate the Low Affinity Leukotriene B₄ Receptor, BLT2*

Received for publication, December 18, 2000, and in revised form, January 17, 2001
Published, JBC Papers in Press, January 18, 2001, DOI 10.1074/jbc.M011361200

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Leukotriene B₄, an arachidonate metabolite, is a potent chemoattractant of leukocytes involved in various inflammatory diseases. Two G-protein-coupled receptors for leukotriene B₄ have been cloned and characterized. BLT1 (Yokomizo, T., Izumi, T., Chang, K., Takuwa, Y., and Shimizu, T. (1997) Nature 387, 620–624) is a high affinity receptor exclusively expressed in leukocytes, and BLT2 (Yokomizo, T., Kato, K., Terawaki, K., Izumi, T., and Shimizu, T. (2000) J. Exp. Med. 192, 421–432) is a low affinity receptor expressed more ubiquitously. Here we report the binding profiles of various BLT antagonists and eicosanoids to either BLT1 or BLT2 using the membrane fractions of Chinese hamster ovary cells stably expressing the receptor. BLT antagonists are grouped into three classes: BLT1-specific U-75302, BLT2-specific LY255283, and BLT1/BLT2 dual-specific ZK 158252 and CP 195543. We also show that 12(S)-hydroxyeicosatetraenoic acid, 12(S)-hydroperoxyeicosatetraenoic acid, and 15(S)-hydroxyeicosatetraenoic acid competed with [³²P]LTB₄ binding to BLT2, but not BLT1, dose dependently. These eicosanoids also cause calcium mobilization and chemotaxis through BLT2, again in contrast to BLT1. These findings suggest that BLT2 functions as a low affinity receptor, with broader ligand specificity for various eicosanoids, and mediates distinct biological and pathophysiological roles from BLT1.

Leukotriene B₄ ((5S,12R)-dihydroxy-6,14-cis-8,10-trans-eicosatetraenoic acid (LTB₄))¹ is a metabolite of arachidonic acid and is one of the most potent activators of granulocytes and macrophages (1–3). BLT, the LTB₄-specific G-protein-coupled receptor (GPCR), is a target for anti-inflammatory drugs, and many BLT antagonists have been developed and are being evaluated. No BLT antagonists are yet approved for clinical use; their lack of efficacy may be due in part to the presence of the other LTB₄ receptors. We cloned BLT1, a high affinity LTB₄ receptor (4), and BLT2, a low affinity LTB₄ receptor (5), and showed that BLT1 is expressed almost exclusively in peripheral leukocytes, whereas BLT2 is expressed ubiquitously with the highest expression in spleen. The structural similarities of these receptors (45% amino acid identity) and the low homologies of BLTs to other known GPCRs suggest that these BLTs comprise a novel receptor family. BLT1 and BLT2 form a gene cluster both in human and mouse genomes. The human BLT2 open reading frame overlaps the promoter region of BLT1 (6), suggesting the expression of these two LTB₄ receptors is tightly intertwined. Human granulocytes, eosinophils, and mononuclear cells express both BLT1 and BLT2 (7), so the precise pharmacological characterization of these two receptors using native cells is difficult. In this paper, we report the inhibitory effects of various BLT antagonists and eicosanoids on LTB₄ binding using CHO cells stably expressing either human BLT1 or BLT2. We also show that several hydroxyeicosanoids other than LTB₄ bind to and activate BLT2 but not BLT1. These findings provide insights into the possible functions of BLT2 and information helpful for the isolation of the other related GPCRs that recognize eicosanoids.

EXPERIMENTAL PROCEDURES

Materials—U75302 (8) and all of the eicosanoids other than LTB₄ were purchased from Cayman Chemical Co. LTB₄ is a generous gift from Ono Pharmaceutical Co. (Osaka, Japan). LY 255283 (9) and LY 223982 (10) were from Lilly Research Laboratories. CP 105696 (11) and CP 195543 (12) are from Pfizer Inc. ZK 158252 is from Schering AG (Berlin, Germany). [³²P]LTB₄ (6956 GBq/mmol) was purchased from PerkinElmer Life Sciences.

Cell Culture and Flow Cytometry—CHO cells stably expressing FLAG-tagged human BLT1 (CHO-FLAG-BLT1) or hemagglutinin (HA)-tagged human BLT2 (CHO-HA-BLT2) were established as described previously (5, 13). The cells were cloned by limiting dilution and their expression of the receptors confirmed by staining the cells with antibodies against the epitope added to the amino terminus of the receptors. The cells were fixed with PBS(−) containing 0.5% (w/v) paraformaldehyde for 5 min on ice and blocked with PBS(−) containing 2% goat serum (Life Technologies, Inc.). The cells were incubated with 30 µg/ml anti-FLAG antibody (clone M5, Eastman Kodak), 10 µg/ml anti-HA antibody (clone CA12-5, Roche Molecular Biochemicals), or 30 µg/ml control mouse IgG (Santa Cruz Biotechnology) in PBS(−) containing 2% oat serum for 30 min at room temperature, followed by staining with 500 × fluorescein isothiocyanate-labeled anti-mouse IgG (Beckman Coulter, CA) for 30 min at room temperature. The cells were washed twice with PBS(−), and analyzed with flow cytometry, Epics XL (Beckman Coulter). Cells expressing each receptor were maintained in Ham’s F12 medium supplemented with 10% fetal calf serum (Sigma), 0.3 mg/ml G418 (Sigma), 100 IU/ml penicillin, and 100 µg/ml streptomycin.

Binding Assay—Cells were harvested and sonicated in a buffer containing...
In contrast, LTB₄ binding to BLT₂ was inhibited by ZK 158252, by measuring the migration, the cells on the upper side of the filter were wiped off and protein (without cremophore) were examined for their binding with 5 nM [³H]LTB₄.

Various BLT antagonists. LTB₄ binding to BLT₁ was inhibited by LY 255283, and CP 195543 but not by 10⁻³ M CP 195543 or LY 223982. We also examined their agonistic activities on these receptors by calcium mobilization and found that 1 and 10⁻³ M U75302 acts as a weak agonist on human BLT₁ (4). On the other hand, the binding of LTB₄ to BLT₂ was concluded that BLT₂ is a low affinity receptor for LTB₄. In the present study, we examined the inhibition by various eicosanoids (Fig. 3) because only LTB₄, 20-hydroxy-LTB₄, and 12(S)-HETE, 12(S)-HPETE, 15(S)-HETE, and 15(S)-HPETE in addition to LTB₄, 20-hydroxy LTB₄, and 12-epi-LTB₄ showed significant competitions at this concentration (Fig. 3A). These results are consistent with our previous results using the membrane fractions of COS-7 cells transiently transfected with BLT₁ (4). On the other hand, the binding of LTB₄ to BLT₂ was inhibited by various eicosanoids (Fig. 3B). 12(R)- and 12(S)-HETE, 12(S)-HPETE, 15(S)-HETE, and 15(S)-HPETE in addition to LTB₄, 20-hydroxy LTB₄, and 12-epi-LTB₄ showed significant inhibition (>50%) of 5 nM [³H]LTB₄ binding. Next, we tested various concentrations of LTB₄, 20-hydroxy-LTB₄, 12(S)-HPETE, 12(S)-HETE, 12(R)-HETE, and 15(S)-HETE in
the competition with [3H]LTB₄ binding to BLT1 and BLT2. Only LTB₄ and 20-hydroxy LTB₄ showed dose-dependent inhibitions of LTB₄ binding to BLT1 (Fig. 4A). In the case of BLT2, however, all of the eicosanoids tested exhibited dose-dependent inhibition of LTB₄, with a rank order of LTB₄ > 12(S)-HETE > 12(S)-HPETE > 12(R)-HETE > 15(S)-HETE > 20-hydroxy LTB₄ (Fig. 4B). These results clearly show that the recognition of the ligands by BLT1 and BLT2 differs, with more promiscuity in BLT2.

Calcium Mobilization by Various Eicosanoids through BLT1 and BLT2—We next examined whether these eicosanoids could activate intracellular signaling through these two receptors. We used a calcium mobilization assay, because it is a very sensitive and quantitative method for detecting LTB₄-BLT interaction. In CHO-FLAG-BLT1 cells, only LTB₄ exhibited a robust increase in intracellular calcium concentrations (Fig. 5A). The one exception was seen in case of 12(S)-HPETE, where there was a very slight signal seen at 10 μM (Fig. 5B). In contrast, all of the ligands that were able to bind to BLT2 (Fig. 4B) exhibited significant increases in intracellular calcium concentrations in CHO-HA-BLT2 cells, showing that these eicosanoids are active on BLT2 (Fig. 5, A and B). Fig. 5, C and D, shows dose-response curves of increase in calcium concentrations on CHO-FLAG-BLT1 and CHO-HA-BLT2 cells, respectively. 12(S)-HETE and 15(S)-HETE up to a concentration of 10 μM did not induce measurable changes in intracellular calcium through BLT1 (Fig. 5C). On the other hand, these eicosanoids exhibited dose-dependent increases in calcium concentrations in CHO-HA-BLT2 cells (Fig. 5D). 12-epi-LTB₄, a 12(S) epimer of LTB₄, acts as a full agonist for BLT1 and BLT2 (Fig. 5, B and C), but a higher concentration of 12-epi-LTB₄ is required. These results clearly show that BLT2 is able to bind to various eico-
A. typical results of calcium mobilizations by various eicosanoids. B, calcium increases in CHO cells by 10 μM eicosanoids (n = 3, average ± S.D.), C and D, dose responses of calcium mobilization by LTB₄, 12(S)-HETE, 15(S)-HETE, and 12-epi-LTB₄ in CHO-FLAG-BLT1 (C) and CHO-HA-BLT2 (D) cells (n = 3, average ± S.D.).
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sanoids and to transduce intracellular signaling.

Chemotactic Response of CHO-BLT2 Cells by Eicosanoids—As LTB₄ is a potent chemoattractant for granulocytes, eosinophils, and macrophages, and CHO cells expressing BLT1 or BLT2 migrate to LTB₄ in vitro (4, 5, 7), we asked whether other eicosanoids could evoke chemotactic responses through BLT1 and BLT2. CHO cells expressing BLT1 showed clear chemotactic activities toward very low concentration of LTB₄ and high concentration (>10 μM) of 12(R)-HETE and 12(S)-HETE, as shown in Fig. 6A. In the case of BLT2, the optimum concentrations of LTB₄ and 12(S)-HETE are close, and the 12(S)-HETE is more effective than 12(R)-HETE (Fig. 6B). The dose-response curves of LTB₄ and 12(S)-HETE in CHO-BLT2 cells are bell-shaped as expected in in vitro chemotaxis assays. 12-epi-LTB₄ also induced chemotaxis both in CHO-BLT1 and CHO-BLT2 cells, with higher optimum concentrations than LTB₄. CHO cells transfected with an empty vector, pcDNA3, as a control did not show any chemotaxis toward LTB₄, 12(S)-HETE, 12(R)-HETE, or 12-epi-LTB₄ (Fig. 6C).

DISCUSSION

LTB₄ is biologically important for the clearance of microorganisms or foreign bodies by activating and recruiting granulocytes to the inflamed lesions (14). Overproduction of LTB₄, however, is also involved in various inflammatory diseases including psoriasis (15), bronchial asthma (16), rheumatoid arthritis (17), ulcerative colitis (18), and ischemic reperfusion injury in various tissues (13). Attempts to understand the biological functions of LTB₄ and to develop potent and selective BLT antagonists would be assisted by the molecular identification of LTB₄ receptors. Several groups including ours have cloned and characterized two distinct LTB₄ receptors, BLT1 and BLT2. BLT1 (4, 19–24), a high affinity and leukocyte-restricted LTB₄ receptor, is presumably the classical BLT, and most of the BLT antagonists developed thus far are targeted to this receptor. Very recently, BLT2, with a structural similarity to BLT1, was identified as a low affinity receptor for LTB₄ (5, 7, 25). One group reported that BLT2 is a high affinity receptor for LTB₄ when expressed in Cos-7 cells (26), but the clear explanations for this discrepancy are not available. The BLT2 gene is localized very close to the BLT1 gene both in human and mouse, suggesting that these two receptors are linked genetically (6, 27). BLT2 shows a relatively ubiquitous expression with the highest level in the spleen, followed by the liver, ovary, and peripheral leukocytes (5, 7). Using semiquantitative reverse transcriptase-polymerase chain reaction analysis, BLT1 and BLT2 have been shown to be coexpressed in human granulocytes, mononuclear cells, and eosinophils (7). A number of papers have reported the inhibitory effects of various BLT antagonists on LTB₄ binding and various LTB₄-induced phenomena, but most of the results were obtained using leukocytes that intrinsically express both BLT1 and BLT2. To correctly understand the pharmacological characters of BLT1 and BLT2, we introduced the expression vectors for these receptors into CHO cells, which do not express any intrinsic LTB₄ receptors, and compared the effects of various BLT antagonists and eicosanoids. These receptors are properly expressed on the cell surface as revealed by flow cytometry (Fig. 1). Binding assays using the fractionated cell membrane revealed clear differences in pharmacological profiles of various BLT antagonists on BLT1 and BLT2. CP 105696 and U75302 effectively inhibited [³H]LTB₄ binding to BLT1, but they did not inhibit LTB₄ binding to BLT2 (Fig. 2), showing that these compounds are specific to BLT1. In addition, U75302 acts as a weak agonist on BLT1, because it increased intracellular calcium in CHO-BLT1 cells (data not shown). On the other hand, LY 255283 inhibited [³H]LTB₄ binding to BLT2 but not to BLT1. CP 195543 and ZK 158252 inhibited [³H]LTB₄ binding to both receptors, but CP 195543 at 1 μM increased intracellular calcium in CHO-BLT2 cells (data not shown). Thus, CP 195543 acts as an antagonist for BLT1 but a weak agonist for BLT2. Various BLT antagonists have been developed and examined for their effects on inflammatory animal models, but no compounds are available for clinical use. If the expression/function of these two receptors are intertwined, as would be predicted based on their juxtaposition in the genome, a clinically important effect may lie in a compound that has independent effects on each receptor as well as one that blocks both. Our studies point to the need to re-evaluate past studies in light of the independent actions of the compound on each receptor and to find a way to screen future candidate compounds.

We next examined the ligand specificity for BLT1 and BLT2. From initial screenings of various eicosanoids on ligand binding assays (Fig. 3), we selected several hydroxy- or hydroperoxyeicosatetraenoic acids to examine carefully the dose-
dependent inhibition of $[^{3}H]$LTB$_4$ binding to BLT1 and BLT2. To our surprise, some of these eicosanoids effectively inhibited LTB$_4$ binding to BLT2 (Fig. 4). The rank order of potency in binding to BLT2 is LTB$_4$ > 12(S)-HETE > 12(R)-HETE > 15(S)-HETE > 20-hydroxy LTB$_4$ (Fig. 4B), which is different from the order of potency in binding to BLT1 (LTB$_4$ > 20-hydroxy-LTB$_4$ => 12(R)-HETE, Fig. 4A (4)). We next examined the agonistic activities of these eicosanoids using a calcium mobilization assay and found that some of them act as agonists on BLT2 (Fig. 5). Although high concentrations of the ligands are required, CHO-BLT2 cells exhibited clear calcium responses toward 1 $\mu$M 12(S)-HETE or 15(S)-HETE. 12(S)-HETE and 12(R)-HETE also induced significant cell migration in CHO-BLT2 cells at lower concentrations than for CHO-BLT1 cells (Fig. 6), suggesting that BLT2 recognizes these eicosanoids and transduces intracellular signaling at physiological concentrations. Despite the broad interests in leukotriene receptors, limited information is available on receptors for HETE and HPETE. Among these receptors, 12(S)-HETE binding sites have been well characterized in human epidermal and carcinoma cells (28–30). High affinity 12(S)-HETE binding sites in human skin were reported, and $K_d$ values for 12(S)-HETE were in the nM range. The rank order of potency in inhibition of $[^{3}H]$12(S)-HETE binding is 12(S)-HETE > 12(R)-HETE ≥ LTB$_4$, which is similar to that of BLT2. In murine B16 melanoma cells, 12(S)-HETE-dependent activation of protein kinase C is mediated by a GPCR and dependent on G$_i$-like G-protein (31). 12(S)-HETE-binding protein in the cytosolic and nuclear fractions of Lewis lung carcinoma cells was characterized and shown to interact as a heterodimer with steroid receptor coactivator-1 (SRC-1) in the presence of 12(S)-HETE (32). The peroxisome proliferator-activated receptor $\alpha$ (PPAR$\alpha$) was also reported to interact with 12(S)-HETE (33). These results suggest that 12(S)-HETE may possess a dual receptor system, cell surface GPCRs, and intranuclear transcriptional factors. BLT2 can interact with high concentrations of 12(S)-HETE, but the $K_d$ of BLT2 for 12(S)-HETE is higher than the reported high affinity 12(S)-HETE receptor(s) in keratinocytes and melanocytes. The selectivity of BLT2 in the recognition of 12(S)-HETE and 15(S)-HETE and the structural information of BLT2 may be helpful for the identification of the high affinity GPCR for HETE.

We also showed by calcium mobilization and chemotaxis assays that 12-epi-LTB$_4$ is active both on BLT1 and BLT2. There are no published reports on the in vivo occurrence or biological functions of 12-epi-LTB$_4$, but it is clear that it acts as an agonist for BLT1 and BLT2 in heterologously expressed systems. Further study is required on the biosynthesis and functions of 12-epi-LTB$_4$.

In summary, we have revealed the specificity of various BLT antagonists on two LTB$_4$ receptors and identified the novel activation of BLT2 by various HETEs and HPETEs. These findings will lead to the development of novel BLT antagonists that may be more specific and therefore more potent as anti-inflammatory drugs, also raising the possibility of the identification of other as yet unknown eicosanoid receptors.

Acknowledgments—We thank Dr. D. Wong (The University of Tokyo) for critically reading this manuscript and S. Ishii, N. Uozumi, and M. Taniguchi (The University of Tokyo) for discussions. We also thank One Pharmaceutical Company for LTB$_4$ and Pfizer Inc., Lilly Research Laboratories, and Schering AG for BLT antagonists.

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