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Transepithelial Migration of Neutrophils in Response to Leukotriene B\(_4\) Is Mediated by a Reactive Oxygen Species-Extracellular Signal-Regulated Kinase-Linked Cascade\(^1\)

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The epithelial cells that form a barrier lining the lung airway are key regulators of neutrophil trafficking into the airway lumen in a variety of lung inflammatory diseases. Although the lipid mediator leukotriene B\(_4\) (LTB\(_4\)) is known to be a principal chemoattractant for recruiting neutrophils to inflamed sites across the airway epithelium, the precise signaling mechanism involved remains largely unknown. In the present study, therefore, we investigated the signaling pathway through which LTB\(_4\) induces transepithelial migration of neutrophils. We found that LTB\(_4\) induces concentration-dependent transmigration of DMSO-differentiated HL-60 neutrophils and human polymorphonuclear neutrophils across A549 human lung epithelium. This effect was mediated via specific LTB\(_4\) receptors and was inhibited by pretreating the cells with N-acetylcysteine (NAC), an oxygen free radical scavenger, with diphenylene iodonium (DPI), an inhibitor of NADPH oxidase-like flavoproteins, or with PD98059, an extracellular signal-regulated kinase (ERK) inhibitor. Consistent with those findings, LTB\(_4\)-induced ERK phosphorylation was completely blocked by pretreating cells with NAC or DPI. Taken together, our observations suggest LTB\(_4\) signaling to transepithelial migration is mediated via generation of reactive oxygen species, which leads to downstream activation of ERK. The physiological relevance of this signaling pathway was demonstrated in BALB/c mice, in which intratracheal instillation of LTB\(_4\) led to acute recruitment of neutrophils into the airway across the lung epithelium. Notably, the response to LTB\(_4\) was blocked by NAC, DPI, PD98059, or CP105696, a specific LTB\(_4\) receptor antagonist. The Journal of Immunology, 2003, 170: 6273–6279.

L \(\text{eukotriene } \text{B}_4\) (LTB\(_4\))\(^3\) is a key mediator of inflammatory processes, immune responses, and host defense against infection (1, 2) and is known to stimulate chemotaxis, degranulation, release of lysosomal enzymes, and reactive oxygen species (ROS) generation (3, 4). Although the precise signaling pathway along which the biological activities of LTB\(_4\) are transduced remains largely unknown, it appears to act via two G protein-coupled receptors: LTB\(_4\) receptor (BLT1), a high affinity receptor first isolated from HL-60 cells, and BLT2, a low affinity receptor that shares ~45% amino acid identity with BLT1 (5–7).

Neutrophils are the primary targets of LTB\(_4\), and their recruitment to sites of inflammation is one of the principal ways in which LTB\(_4\) contributes to inflammatory responses. In particular, the influx of neutrophils into the airway is a common feature of several inflammatory diseases, including acute bronchitis, adult respiratory distress syndrome (ARDS), and chronic obstructive pulmonary disease (COPD) (8, 9), as well as severe asthma (10, 11). Once leukocytes are beyond the endothelial barrier, chemotactic signals frequently lead to their accumulation near mucosal epithelial cells and in the lumen of the airway. Airway epithelial cells may thus be important regulators not only of retention and activation of leukocytes, but also of their passage into the airway itself. It has therefore been suggested that LTB\(_4\)-induced transepithelial migration of neutrophils plays a crucial role in the pathogenesis of ARDS, COPD, and severe asthma. Yet despite this implication, the signaling pathway via which LTB\(_4\) mediates transepithelial migration of neutrophils remains largely unknown.

In the present study, therefore, we investigated the signaling pathway by which LTB\(_4\) induces transepithelial migration of neutrophil-like granulocytes (differentiated HL-60 neutrophils (dHL-60)) differentiated from HL-60 cells, which have been shown to be a valid model system for the analysis of human neutrophilic migration and chemotaxis (12), and by human polymorphonuclear neutrophils (PMNs) isolated from healthy volunteers. Our results indicate that LTB\(_4\)-induced transepithelial migration is accomplished via an ROS-extracellular signal-regulated kinase (ERK)-linked pathway. In addition, the physiological relevance of this pathway was validated in vivo by demonstrating that ROS generation and ERK activation are both required for transmigration of peripheral blood neutrophils across the airway epithelium of BALB/c mice.

Materials and Methods

Chemicals

2,7'-Dichlorofluorescin diacetate (DCFDA) was purchased from Molecular Probes (Eugene, OR). LTB\(_4\)APA, LY1718, genistein, and AACOCF3
were obtained from Biomol (Plymouth Meeting, PA). LTβ, and U75302 were purchased from Cayman Chemical Co. (Ann Arbor, MI). BSA, DMSO, PMA, GFI09203X, wortmannin, diphenylethyl iodonium (DPI), and N-acetylcysteine (NAC) were obtained from Sigma-Aldrich (St. Louis, MO). FBS, DMEM, phenol red-free DMEM, gentamicin, and nonessential amino acids were obtained from Life Technologies (Gaithersburg, MD). All other chemicals were obtained from standard sources and were molecular biology grade or higher.

Cell culture and differentiation
Human promyelocytic HL-60 cells obtained from American Type Culture Collection (Manassas, VA; CCL-240) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 0.1 mM nonessential amino acids, 50 μM of β-mercaptoethanol, and 50 μg/ml streptomycin at 37°C under a humidified 95%/5% (v/v) mixture of air and CO₂. To induce cells to undergo differentiation along the neutrophil lineage, aliquots of HL-60 cell suspension (5 × 10⁵ cells/ml) were seeded onto dishes and grown for 5 days in the absence or the presence of 1.25% DMSO. Cell differentiation was then evaluated using four criteria (13–15): 1) morphological changes (nuclear segmentation and granules formation); 2) cell proliferation was assayed using H&E cytochemical staining; 2) cell proliferation was assayed with 0.4% trypan blue exclusion; 3) the capacity to reduce nitro blue tetrazolium, a property associated with myeloid cell maturation, was determined spectrophotometrically at 540 nm; and 4) surface expression of differentiation-related Ags was evaluated by flow cytometry using FITC-conjugated mAbs against CD11b, CD16, and CD33. A549 human lung epithelial cells were grown in RPMI containing 10% FBS, 0.1 mM non-essential amino acids, 50 μM of β-mercaptoethanol, and 100 μg/ml streptomycin. Human PMNs were isolated from blood samples collected from healthy volunteers in heparin sodium salt (100 U; Sigma-Aldrich; H-3149). PMNs were separated on a Ficoll-Paque density gradient (1.077 ± 0.001 g/ml), followed by 2% dextran sedimentation to remove the majority of RBCs (Amer-then incubated for 10 min with the H₂O₂-sensitive DCF. After incubation with DCFDA, cells were exposed to LTB₄ or PMA for 20 min before the addition of LTB₄. C cells in each dish were then treated with LTβR or control buffer for 6 h, after which they were harvested, lysed in 0.1 ml of lysis solution (0.2 M Tris-Cl (pH 7.6) and 0.1% Triton X-100), and spun for 1 h. The amount of DNA used in each transfection was held constant (1.8 μg) by adding sonicated calf thymus DNA. To measure ERK’s kinase activity using a PathDetect trans-reporting system (catalog no. 219005; Stratagene), the cells were grown for 24 h in fresh RPMI 1640 supplemented with 10% FBS for an additional 2 h. The cells in each dish were then treated with LTβR or control buffer for 6 h, after which they were harvested, lysed in 0.1 ml of lysis solution (0.2 M Tris-Cl (pH 7.6) and 0.1% Triton X-100), and spun for 1 h. The resultant supernatants were assayed for protein and β-galactosidase activity. Luciferase activity was assayed in 10-μl samples of extract. Luciferase luminescence was counted in a luminometer (Turner Design, Sunnyvale, CA; TD-20/20) and normalized to cotransfected β-galactosidase activity (16). Transfection experiments were performed in triplicate using two independently isolated sets of cells, and the results were averaged.

LTβR-challenged mouse model
Pathogen-free female BALB/c mice (8 wk old) were purchased from the Korea Research Institute of Chemistry Technology (Daejon, Korea). The mice were housed throughout the experiments in a laminar flow cabinet and were provided with standard laboratory chow and water ad libitum. For intratracheal administration, mice were anesthetized by i.p. injection of 150 μl of saline containing 13 mg/ml ketamine HCl (Yuhan, Seoul, Korea) and 88.0 μg/ml xylazine (Bayer, Seoul, Korea). LTβ (Cayman Chemicals, Ann Arbor, MI) was prepared in PBS containing 20% ethanol to a final concentration of 20 μg/ml, and 50 μl was injected intratracheally. After 6 h, bronchoalveolar lavage (BAL) fluid was collected from the lung space as previously described (17). Briefly, the chest cavity was exposed to allow for expansion, after which the trachea was carefully intubated, and the catheter was secured with ligatures. Eight hundred microliters of pre-warmed 1% NaCl solution was slowly infused into the lung and withdrawn. The aliquots were then pooled and then kept at 4°C. To obtain differential cell counts, the BAL fluid was cytospun and stained with Diff-Quik (Dade Diagnostics, Aguada, Puerto Rico). Two independent, blinded investigators then counted the cells using a hemocytometer. Approximately 400 cells were counted in each of four randomly selected locations. The percentage of neutrophils was multiplied by the total cell number to obtain the neutrophil count. To evaluate lung histology, the lungs were removed.
from mice 6 h after LTB4 challenge, cut into sections, and stained with H&E (Sigma-Aldrich) (18).

Data analysis and statistics

Data are expressed as mean percentages of control ± SD. Statistical comparisons between groups were made using Student’s t tests. Values of p < 0.01 were considered significant.

Results

**LTB4-induced transepithelial migration of dHL-60 cells**

Human promyelocytic HL-60 cells induced with DMSO to differentiate toward neutrophil-like granulocytes (dHL-60) have been shown to be a valid model system for the analysis of human neutrophil migration and chemotaxis, (12, 19, 20). In the present study LTB4 induced migration of dHL-60 cells, but not the parental HL-60 cells, across a monolayer of A549 human type II alveolar epithelial cells (Fig. 1). The effect was concentration dependent, with 2.3-fold more migration occurring in the presence of 100 nM LTB4 than control buffer.

Previous reports have shown that the number of BLTs increases during DMSO-induced differentiation of HL-60 cells (6, 20, 21). Consistent with those reports, RT-PCR showed little or no expression of BLT1 or -2 in parent HL-60 cells, but their expression increased with maturation toward the neutrophil lineage; levels of BLT2, in particular, were dramatically increased during maturation (Fig. 2A).

With that in mind, we next tested whether the ability of dHL-60 cells to migrate across lung epithelium was a specific result of the increased expression of BLTs by examining the effects of selective BLT antagonists. As shown in Fig. 2B, pretreatment with either U75302 (5), a selective BLT1 antagonist, or LTB4 APA, a nonspecific BLT antagonist, significantly diminished LTB4 (100 nM)-induced transepithelial migration of dHL-60 cells, and in combination, they abolished the response. That LTB4 APA exerted a more profound inhibitory effect than U75302 suggests that the effect was mediated primarily by BLT2. Consistent with that idea, LTB4 showed biological activity at concentrations of 100–500 nM, which falls within the reported optimal range for BLT2 and is 2 orders of magnitude higher than the optimal range for BLT1. On the other hand, pretreatment with LY1718, a specific cysteinyl LT antagonist, had no inhibitory effect (Fig. 2B).

**ROS is critical for the transepithelial migration of dHL-60 cells**

Recently, a ROS-dependent pathway was shown to mediate LTB4-induced chemotaxis of Rat-2 fibroblasts (22). To determine whether ROS is similarly involved in the transepithelial migration of dHL-60 cells induced by LTB4, we examined the effect on LTB4-mediated transepithelial migration of ROS inhibition by DPI, an inhibitor of NADPH oxidase-like flavoenzymes, or by NAC, a ROS scavenger. We found that transmigration induced by 100 nM LTB4 was significantly diminished by pretreating dHL-60 cells with DPI and NAC (Fig. 3A). In a similar fashion, pretreatment with DPI or NAC also diminished transmigration induced by PMA. To determine whether LTB4 in fact triggers intracellular ROS generation in dHL-60 cells, we assessed H2O2 levels using DCF. As shown in Fig. 3B, LTB4 elicited a concentration-dependent increase in the level of ROS, and this effect was abolished by pretreating the cells with DPI or NAC (Fig. 3C).

**LTB4-induced transepithelial migration of dHL-60 cells requires ERK activation**

We also observed that in dHL-60 cells LTB4 (100 nM) induced phosphorylation of ERK (Fig. 4A), but not p38 or c-Jun amino-terminal kinase (not shown). To further evaluate ERK stimulation by LTB4, ERK’s kinase activity was assessed using an Elk-luciferase trans-reporter system. We found that LTB4 (100 nM) clearly enhanced ERK/Elk-luciferase activity in differentiated dHL-60 cells, but not in wild-type HL-60 cells (Fig. 4B). Moreover, pretreating the cells with 10 or 20 μM PD98059, a specific mitogen-activated protein kinase kinase inhibitor that blocks ERK activation, dose-dependently inhibited the transepithelial migration induced by 100 nM LTB4 (Fig. 4C). Pretreatment with genistein (10 μM), a nonspecific tyrosine kinase inhibitor, had a similar inhibitory effect.

In a subsequent experiment aimed at determining whether ERK activation is situated upstream or downstream of ROS generation in the LTB4 signaling pathway, we tested the effects of DPI and NAC on LTB4-induced ERK phosphorylation. As shown in Fig. 4D, pretreatment with either NAC or DPI virtually abolished LTB4-induced ERK phosphorylation. Apparently, LTB4 signaling to transmigration of dHL-60 cells is transduced via ERK, most likely acting downstream of ROS.

We further investigated the mediators contributing to LTB4 signaling by testing the effects of inhibitors of phosphatidylinositol 3-kinase (PI 3-kinase), protein kinase C (PKC), and cytosolic phospholipase A2 (cPLA2) on transepithelial migration and ERK
activation. We found that LTB$_4$-induced transmigration was completely blocked by pretreatment with 0.1 $\mu$M wortmannin, a PI 3-kinase inhibitor, or 0.1 $\mu$M GF109203X, a PKC inhibitor (Fig. 5A), and there was a corresponding decrease in the level of ERK activation (Fig. 5B), which suggests that both PI 3-kinase and PKC are situated upstream of ERK in the LTB$_4$ signaling pathway. By contrast, AACOCF3 (10 $\mu$M), a specific cPLA$_2$ inhibitor, had no effect on either transmigration or ERK activation (Fig. 5).

A ROS-ERK-linked cascade is essential for LTB$_4$-induced transepithelial migration of PMNs

The functional importance of this ROS-ERK-linked pathway was further confirmed by investigating its presence in human PMNs obtained from peripheral blood of healthy volunteers. As in dHL-60 cells, LTB$_4$ induced significant transepithelial migration of human PMNs, and the effect was significantly diminished by pretreatment with NAC, DPI, or PD98059 (Fig. 6).

Roles of ROS and ERK in migration of neutrophils across airway epithelium in LTB$_4$-challenged mice

To investigate the physiological function of ROS-ERK-linked signaling in vivo, BALB/c mice were subjected to intratracheal instillation of LTB$_4$. Analysis of BAL fluid revealed that very few neutrophils were present in the airways following intratracheal instillation of saline (Fig. 7); mostly alveolar macrophages were detected. Following instillation of 1 $\mu$g of LTB$_4$ in 50 $\mu$l of saline,...

FIGURE 3. Role of ROS in LTB$_4$-induced transepithelial migration. A, dHL-60 cells were pretreated with 5 $\mu$M DPI or 2 mM NAC for 20 min at room temperature before adding 100 nM LTB$_4$ to the lower Transwell chamber. Transmigrated cells were counted, and the results are expressed as the mean percentage of control $\pm$ SD. B, Serum-starved dHL-60 cells were incubated for 5 min with the indicated concentration of LTB$_4$ or 20 ng/ml PMA, after which DCF fluorescence was quantified. Data are expressed as the mean percentage of control $\pm$ SD from three independent experiments. C, Serum-starved dHL-60 cells were exposed to 100 nM LTB$_4$ for 5 min in the presence or the absence of 5 $\mu$M DPI or 2 mM NAC. Inhibitors were added 20 min before the addition of LTB$_4$. DCF fluorescence was quantified as described in Materials and Methods. Data are expressed as the mean percentage of control $\pm$ SD from three independent experiments.

FIGURE 4. Role of ERK activation in LTB$_4$-induced transepithelial migration. A, HL-60 (day 0) or dHL-60 cells (days 3 and 5) were treated with 100 nM LTB$_4$ for 5 min, after which the lysates were immunoblotted with anti-phospho-ERK Ab. The blot was then stripped and reprobed with anti-total ERK Ab. The results shown are representative of at least three independent experiments. B, ERK kinase activity was measured using the Elk-luciferase trans-reporter system as described in Materials and Methods. HL-60 or dHL-60 cells were transiently cotransfected with pFR-Luc reporter plasmid and then treated with 100 nM LTB$_4$ or control buffer for 6 h. The lysates were assayed for Elk-luciferase activity. Data are expressed as the mean percentage of control $\pm$ SD from three independent experiments. C, Effect of ERK inhibition on transepithelial migration of dHL-60 cells. For the inhibitor experiments dHL-60 cells were pretreated for 20 min with control buffer, 10 $\mu$M genistein, or the indicated concentration of PD98059 before adding 100 nM LTB$_4$ to the lower Transwell chamber. After 6 h the transmigrated cells were stained with 0.4% trypan blue and counted. Data are expressed as the mean percentage of control $\pm$ SD from three independent experiments. D, dHL-60 cells were treated with 100 nM LTB$_4$ for 5 min in the presence or the absence of DPI (5 $\mu$M) or NAC (2 mM) and then lysed. The lysates were immunoblotted with anti-phospho-ERKs Ab, after which the blot was stripped and reprobed with anti-total ERKs Ab. The results shown are representative of at least three independent experiments.
however, the number of neutrophils in the BAL fluid increased significantly within 6 h (Fig. 7, A and B). This effect was blocked by prior i.p. administration of NAC, DPI, PD98059, or the BLT inhibitor CP105696 (Fig. 7C), suggesting that in vivo a ROS-ERK-linked signaling pathway governs LTB4-mediated neutrophil trafficking into the airway.

Discussion
The epithelial cells that form a barrier lining the airway of the lung are key regulators of neutrophil trafficking into the airway lumen. In that regard, LTB4 is known to be among the most potent of endogenous chemoattractants of PMNs (along with IL-8 and C5a) and to stimulate adhesion of PMNs to airway epithelial cells and transmigration. However, despite its role as a major mediator of PMN transepithelial migration and neutrophil-induced lung injury, little is known about the LTB4 signaling cascade involved in these processes. The major observation of the present study is that a ROS-ERK-linked signaling pathway mediates LTB4-induced transepithelial migration of neutrophils both in vitro and in vivo.

A growing body of evidence suggests that oxygen-derived radicals (e.g., O2•− and H2O2) serve as intracellular signaling molecules for a variety of agonists, including LTB4, TNF-α (16, 23, 24), IL-1 (25), and TGF-β1 (27), as well as various growth factors (27–30). In the context of the present study it is notable that a ROS-linked cascade was recently shown to mediate LTB4-induced chemotaxis and proliferation of Rat-2 fibroblasts (22), that oxidant stress generated by LTB4 reportedly induces transendothelial migration of monocytes (31, 32), and that ROS reportedly contribute to integrin-dependent cell adhesion (33, 34). Given the inhibitory effect of DPI on LTB4-induced cell migration and ERK activation, we suggest that NADPH oxidase is the primary source of the ROS generated in response to LTB4. Consistent with that idea, several studies have shown that LTB4 promotes robust, receptor-mediated activation of NADPH oxidase (35–37), which in eosinophils mediates activation of PKC, phospholipases C and D, and cPLA2 (35, 38).

Neutrophils function in the front line of cellular host defense against microorganisms. This function relies in part on their ability to generate large amounts of O2•− and related ROS through activation of NADPH oxidase on the plasma membrane, a phenomenon known as respiratory bursting (14, 36, 39). Thus, ROS generation by NADPH oxidase appears to have a dual function, host defense and intracellular signaling, although it remains unknown how the two functions are respectively regulated. The fact that the level of ROS generated by LTB4 is substantially lower than those seen during respiratory bursts suggests that the regulatory mechanisms are quite different.

We were able to demonstrate that ERK mediates ROS signaling to transepithelial migration of neutrophils both in vitro and in vivo (Figs. 4 and 7). In vivo, the process of transepithelial neutrophil migration occurs through a series of steps orchestrated by the interaction of adhesion molecules on the surface of the neutrophils and proteins secreted by the bronchial epithelial cells. Inhibition of ROS or ERK may interfere with any one or more of these steps.

Recently, we and others reported that PI 3-kinase acts as an upstream mediator of ROS generation, leading to chemotaxis and ERK activation (40, 41). Herein we showed that pretreating dHL-60 cells with wortmannin or GF109203X, inhibitors of PI 3-kinase and PKC, respectively, diminished both LTB4-induced transmigration and ERK phosphorylation, suggesting that PI 3-kinase and PKC act upstream of ERK to regulate LTB4 signaling. Consistent with that idea, PMA, an activator of several PKC isoforms, stimulated transepithelial migration of dHL-60 cells, and the effect was significantly diminished by pretreatment with antioxidants.

ROS generation in response to external stimuli has been related to the activation of transcription factors AP-1 and NF-κB (34),
which appear to be involved in the stimulation of the integrins LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18). Although neutrophil adherence and subsequent migration out of postcapillary venules during systemic inflammation are largely dependent on CD18, the existence of CD18-independent neutrophil migration has also been demonstrated (42–45). For example, when Doerschuk and co-workers (43) compared neutrophil migration in the systemic and pulmonary circulations of rabbits exposed to HCl, Streptococcus pneumoniae, E. coli endotoxin, or PMA, they found that migration into the abdominal wall was always CD18-dependent, but migration into the lung was not. Similarly, analysis of neutrophil migration into the inflamed joints of rats with adjuvant arthritis showed that migration could only be completely blocked by combining an anti-CD18 Ab with an Ab recognizing β1 very late Ag 4 (CD49d/CD29). Likewise, monocytes are capable of CD18-independent migration mediated in part by members of the β1 integrin family, very late Ag 4 in particular (44, 45). In addition to integrins, chemokines and proteases, such as elastase, would also seem to be likely downstream targets of ROS-ERK-linked signaling. With further study we anticipate complete clarification of the molecular linkage between the ROS-ERK-linked pathway and its various potential targets. Considering a critical role of LTB4 as a major mediator of PMN transepithelial migration and neutrophil-induced lung injury, specific blockade of the ROS-ERK-linked pathway may have therapeutic value in the treatment of inflammatory diseases such as acute bronchitis, ARDS, COPD, and severe asthma.

References