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Transepithelial Migration of Neutrophils in Response to Leukotriene B_4 is Mediated by a Reactive Oxygen Species-ERK-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 (dHL-60) and human polymorphonuclear neutrophils across A549 human lung epithelium. This effect was mediated via specific LTB_4 receptors (BLT) and was inhibited by pretreating the cells with NAC, an oxygen free radical scavenger, with DPI, an inhibitor of NADPH oxidase-like flavoproteins, or with PD98059, an 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 (ROS), which leads to downstream activation of ERK. The physiological relevance this signaling pathway was demonstrated in BALB/c mice, where 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 BLT antagonist.

Materials and Methods

Transepithelial migration assays

Transepithelial migration assays were carried out as previously described (8). Briefly, a collagen-coated, polycarbonate filter insert (5 μm pore size) was placed inside each well of several Transwell 12-well tissue culture plates to create a well within a well, after which 5.2 ml of medium was added to completely submerge the insert. Using sterilized forceps, the insert was then inverted, ensuring that no air bubbles were trapped under it, and exactly 0.8 ml of medium was removed from each well. The insert was then moved to the center of its well to avoid an overlapping meniscus at the edge. Each inverted insert was then gently seeded with a total of 0.5×10^6 A549 cells in two 50-μl aliquots of cell suspension, yielding a configuration in which the cells were growing on the microporous membrane with air above and medium below. The plates were then incubated at 37°C under a 5% CO_2 atmosphere for 4 to 5 days, at which time the inserts were returned to their original orientation for the transmigration experiments. For experimentation, HL-60 cells or PMNs were seeded into the upper chambers of the Transwell system to a density of 5×10^5/well, and the indicated concentrations of LTB_4 or saline were added to the lower chambers. After incubating at 37°C in 95%/5% (v/v) mixture of air and CO_2 for 6 h, the amount of basal-to-apical transepithelial migration of dHL-60 or human PMNs was determined by counting the migrated cells on the underside of the insert. When assessing the effects of inhibitors, cells were pretreated with the inhibitor of interest for 20 min prior to the addition of LTB_4.

Measurement of intracellular H_2O_2

Intracellular H_2O_2 was measured as a function of DCF fluorescence using a fluorometer. Briefly, HL-60 cells were grown for 5 days in the presence of 1.25% DMSO and then serum-starved for 6 h in phenol-red free RPMI supplemented 0.5% FBS. To measure intracellular H_2O_2, cells were then incubated for 10 min with the H_2O_2-sensitive fluorophore DCFDA (5 μg/ml), which when taken up fluorescently labels intracellular H_2O_2 with DCF. After incubation with DCFDA, cells were exposed to LTB_4 or PMA for 5 min and immediately observed under a fluorometer from Molecular Devices (spectraMAX geminiXS, USA). DCF fluorescence was excited at 488 nm, and the evoked emission was filtered with a 515 nm ling pass filter.

Results

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

Recently, a ROS-dependent pathway was shown to mediate LTB_4-induced chemotaxis of Rat-2 fibroblasts (20). To determine whether ROS is similarly involved in the transepithelial migration of dHL-60 cells induced by LTB_4, we examined the effect on LTB_4-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 LTB_4 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 LTB_4 in fact triggers intracellular ROS generation in dHL-60 cells, we assessed H_2O_2 levels using DCF. As shown in Figure 3B, LTB_4 elicited a concentration-dependent increase in the levels of ROS, and this effect was abolished by pretreating the cells with DPI or NAC (Fig. 3C).

LTB_4-induced transepithelial migration of dHL-60 cells requires ERK activation

We also observed that in dHL-60 cells LTB_4 (100 nM) induced phosphorylation of ERK (Fig. 4A), but not p38 or JNK (not shown). To further evaluate ERK stimulation by LTB_4, ERK's kinase activity was assessed using an Elk-luciferase trans-reporter system. We found that LTB_4 (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 MEK inhibitor that blocks ERK activation, dose-dependently inhibited the transepithelial migration induced by 100 nM LTB_4 (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 LTB_4 signaling pathway, we tested the effects of DPI and NAC on LTB_4-induced ERK phosphorylation. As shown in Fig. 4D, pretreatment with either NAC or DPI virtually abolished LTB_4-induced ERK phosphorylation. Apparently, LTB_4 signaling to transmigration of dHL-60 cells is transduced via ERK, most likely acting downstream of ROS.

We further investigated the mediators contributing to LTB_4 signaling by testing the effects of inhibitors of PI 3-kinase, PKC and $cPLA_2$ on transepithelial migration and ERK activation.

We found that LTB_4-induced transmigration was completely blocked by pretreatment with 0.1 μM wortmannin, a PI 3-kinase inhibitor, or with 0.1 μM 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 μM), a specific $cPLA_2$ inhibitor, had no effect on either transmigration or ERK activation (Fig. 5).