Cysteinyl Leukotriene-1 Receptor Activation in a Human Bronchial Epithelial Cell Line Leads to Signal Transducer and Activator of Transcription 1-Mediated Eosinophil Adhesion

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ABSTRACT

We studied the effect of leukotriene D4 (LTD4) on a human bronchial epithelial cell line (16HBE) overexpressing the cysteinyl leukotriene (CysLT) 1 receptor (HBECysLT1R), looking at the associated signal transduction mechanisms as well as at effects on inflammatory cell adhesion. The results obtained showed that LTD4 increases the phosphorylation of extracellular signal-regulated protein kinase (ERK) 1/2 and of the signal transducer and activator of transcription 1 (STAT-1) in serine 727 (STAT-1Ser727), resulting in increased eosinophil adhesion to HBECysLT1R, associated with enhanced surface expression of intercellular adhesion molecule (ICAM) 1. Pretreatment with a CysLT1R-selective antagonist or with a selective inhibitor of protein kinase C (PKC) or with a selective inhibitor of the mitogen-activated protein kinase kinase (MEK) successfully suppressed both LTD4-induced STAT-1Ser727 phosphorylation and the associated increase in eosinophil adhesion. The use of the MEK inhibitor and of the selective CysLT1R antagonist in electrophoretic mobility shift assay experiments showed that LTD4 promotes the nuclear translocation of STAT-1 through the activation of ERK1/2 pathway. The key role of STAT-1 in leukotriene D4 transduction signaling was confirmed by RNA interference experiments, where silencing of STAT-1 expression abolished the effect of leukotriene D4 on eosinophil adhesion. In conclusion, for the first time, we provide evidence of the involvement of STAT-1 in the signal transduction mechanism of the CysLT1 receptor, phosphorylation of STAT-1, through PKC and ERK1/2 activation, causes enhanced ICAM-1 surface expression and eosinophil adhesion. Effective CysLT1R antagonism may therefore contribute to the control of the chronic inflammatory condition that characterizes human airways in asthma.

Cysteinyl leukotrienes (CysLTs) play an important role in the pathogenesis of airway inflammation and remodeling in asthma (Drazen, 1998; Bisgaard, 2000; Henderson et al., 2002). The biological effects of CysLTs are mediated by at least two G protein-coupled receptors (GPCRs), namely cysteinyl leukotrienes receptor 1 (CysLT1R) and receptor 2, and the CysLT1R is known to be involved in most of the biological effects in the lung (Nicosia et al., 1999; Dahlén, 2000). CysLT1R is expressed in smooth muscle cells and lung macrophages (Lynch et al., 1999) and is widely distributed in human eosinophils, monocytes, and neutrophils (Figueroa et al., 2001; Mita et al., 2001); little is known about the expression and the responses of CysLT1R in the airway epithelium, but recent evidence showed that LT/C4 may elicit transform-
ing growth factor β release via the activation of p38 kinase pathway in human airway epithelial cells (Perg et al., 2006).

Leukotriene D4 (LTD4) has been reported to activate the mitogen-activated protein kinases (MAPKs) p38 and the extracellular signal-regulated kinase (ERK) 1/2, through phosphatidylinositol 3-kinase and protein kinase C (PKC) activation in human renal mesangial cells (McMahon et al., 2000); furthermore, activation of the ERK1/2 through a PKCα-Raf-1-dependent pathway has also been reported in a human monocytic leukemia cell line (Hoshino et al., 1998). Studies performed in intestinal epithelial cells demonstrated that LTD4 activates the proliferative response through a distinct Ras-independent and PKC-dependent ERK1/2 activation (Paruchuri et al., 2002), migration through a phosphatidylinositol 3 kinase and Rac-dependent mechanism (Paruchuri et al., 2005), and stress fiber formation by a RhoA and PKC-dependent mechanism (Massoumi et al., 2002).

The signal transducer and activator of transcription (STAT) 1 pathway has been associated with the pathogenesis of asthma (Chen et al., 2004; Quarcoo et al., 2004), and it is known to be involved in interferon (IFN)-γ signaling pathway (Darnell, 1997). Within the sequence of STAT, the C-terminal Ser727, located within a potential MAPK consensus PMSP motif, is phosphorylated by an unknown kinase, and this event increases the transcription factor activity of STAT-1 (Wen et al., 1995; Darnell, 1997). Several GPCRs, such as angiotensin II, AT1, and thrombin receptors, can regulate STAT-1 activity through the interaction between ERK1/2 activity and STAT-1 serine phosphorylation (Schindler and Darnell, 1995), suggesting that LTD4 may also have this activity.

To test the possible involvement of STAT-1 in the transcription mechanisms associated with the activation of the CysLT1 receptor, we investigated the effects of LTD4 in a transformed human bronchial epithelial cell line (16HBE) overexpressing the CysLT1 receptor (HEBCysLT1R), reporting that LTD4 increases the PKC-dependent activation of ERK1/2 and STAT-1 pathways, leading to increased intracellular adhesion molecule (ICAM) 1 expression and eosinophil adhesion.

Materials and Methods

Transfection and Transfection of pBH-CysLT1R Construct in Epithelial Cells. The SV40 large T antigen-transformed human airway epithelial cell line (16HBE) was used for these studies. The 16HBE cell line was cultured as an adherent monolayer in Eagle’s minimum essential medium supplemented with 10% heat-inactivated (56°C, 30 min) fetal calf serum + 100 U/ml penicillin and 100 mg/ml streptomycin. 16HBE cells have previously been used to study the functional properties of bronchial epithelial cells in inflammation (Merendino et al., 2006). Because 16HBE cells have been showing a weak and variable expression of cysLT1R, we enhanced and normalized its expression by transfection and transfection with a pBH-CysLT1R construct. CysLT1R cDNA was obtained from pDNA 3.1 plasmid (Merck and Co., Inc. Research Laboratories, West Point, PA) (Lynch et al., 1999) using a PCR reaction with the primers LT1 EcoRI 5'-GGA ATT CAC CAT GGA TGA AAC AGG AAA TCT GAC AG-3' and LT1 SalI 5'-ACG CCT CGA CCT ATT TAC ATA TTT CTG CTC TCT TTT-3' (Invitrogen, Carlsbad, CA). The PCR product, after EcoRI and SalI digestion, was subcloned into the EcoRI SalI site of the retroviral vector pBH to allow stable expression in infected cells.

Phoenix cells were plated at 3 to 3.5 million cells per 100-mm plate. After 24 h, 10 μg of DNA (pBH-CysLT1R) were added following a CaCl2 transfection protocol (Graham and van der Eb, 1973). The following day the viral supernantant was filtered, and 5 ml was added to 16HBE plates. Forty-eight hours post-transfection, hygromycin (50 μg/ml) was added to isolate resistant cells (HEBCysLT1R cells). The expression of the cysLT1R was routinely checked by Western blot analysis using a commercially available antibody (Cayman Chemical, Ann Arbor, MI), showing a sustained and reproducible expression (data not shown).

Stimulation of HEBCysLT1R. Viable HEBCysLT1R cells (0.5 × 10⁶) were plated into 75-cm² flasks with RPMI 10% FBS for 72 h; confluent cells were maintained for 24 h in RPMI without FBS and were stimulated with LTD4 (0.01–1 μM) (Sigma-Aldrich, St. Louis, MO), in the presence or absence of Montelukast (Merck and Co., Inc. Research Laboratories; 0.1–1 μM), added 1 h before LTD4 stimulation.

LTD4 stimulation was also carried out in the presence of IFN-γ (R&D Systems, Minneapolis, MN) using the cells treated with LTD4 (0.1 μM) for 24 h (adhesion tests) or 15 min (signaling) in the presence or absence of IFN-γ (50 ng/ml), added 1 h before LTD4 stimulation. The involvement of specific intracellular signaling pathways was evaluated pretreating the cells with GF109203X (a PKC inhibitor, 10 μM; Sigma-Aldrich) or PD98059 (an MEK inhibitor, 25 μM; Sigma-Aldrich).

Eosinophil Separation and Adhesion Assay. Peripheral blood eosinophils were prepared from normal subjects with the use of dextran sedimentation and centrifugation for Ficoll cushions, as described previously, followed by negative immunomagnetic selection (Profiti et al., 2003). Eosinophil adhesion was performed as previously described with minor modifications (Zeidler et al., 2000). Purified eosinophils were resuspended in PBS (10⁶ cells/ml), labeled for 45 min at 37°C with 50 μg/ml the fluorochrome dye SFDA (Invitrogen), washed, and resuspended in PBS (0.4 × 10⁶ cells/ml). Immediately before addition of eosinophils, medium was removed from the HEBCysLT1R cultures (70,000 HEBCysLT1R cells/well) grown to confluence in standard 24-well culture plates in complete medium, and cells were washed with warm PBS. Labeled eosinophils (0.2 × 10⁶ cells/well) were added in a final volume of 0.5 ml. The plates were incubated at 37°C for 25 min, and total fluorescence was evaluated using an excitation wavelength of 485 nm and monitoring emission at 530 nm in a Wallac 1420 Victor multilabel counter (PerkinElmer Life and Analytical Sciences-Wallac Oy, Turku, Finland). Later, nonadherent cells were removed by washing, and fluorescence was measured to evaluate bound cells. Adhesion was expressed as percentage of the fluorescence ratio of bound cells on total cells. All test points were performed in triplicate.

Identification of ICAM-1 Expression. The expression of ICAM-1 on the surface of HEBCysLT1R cells was determined by direct label immunofluorescence using a FACSStar Plus (BD Biosciences, San Jose, CA) analyzer. A conjugated mouse anti-human ICAM-1 (anti-CD54, clone 6.5B5) (Dako Denmark A/S, Glostrup, Denmark) that react with fluorescence was measured to evaluate bound cells. Adhesion was expressed as percentage of the fluorescence ratio of bound cells on total cells. All test points were performed in triplicate.

Western Blotting. Total proteins were extracted from HEBCysLT1R using radioimmunoprecipitation assay buffer (1 × PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 mM Na2VO4, 10 μg/ml PMSF), and the phosphorylation of p38 MAPK, of ERK1/2 (Cell Signaling Technology Inc., Danvers, MA), and of STAT-1 (Ser727 and Tyr710), as well as the total amount of p91/STAT-1α (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was evaluated using specific antibodies. β-Actin (Sigma-Aldrich) was used as a housekeeping protein to control the total amount of protein in each sample.

Cell extracts were transferred in microcentrifuge tubes, left on ice for 45 min, and centrifuged at 15,000 g for 20 min at 4°C. Sixty micrograms of proteins was subjected to SDS-polyacrylamide gel electrophoresis on 4 to 12% gradient gels and blotted onto nitrocel-
abolished by pretreating epithelial cells with the CysLT1R antagonist Montelukast (0.1–10 μM) inhibited in a concentration-dependent fashion the effect of LTD4 with maximum response at concentrations ≥ 0.1 μM (Fig. 1A). As expected, the specific CysLT1R antagonist Montelukast (0.1–10 μM) inhibited in a concentration-dependent fashion the effect of LTD4 (0.1 μM) with maximal response at concentrations ≥ 1 μM (Fig. 1B). The increase in eosinophil adhesion was accompanied by a statistically significant increase in the expression of ICAM-1 on the surface of HBECysLT1R cells (Fig. 2), as shown by fluorescence-activated cell sorting analysis of cells incubated with increasing concentrations of LTD4; as expected, also this effect was abolished by pretreating epithelial cells with the CysLT1R antagonist Montelukast (1 μM; data not shown).

**Preparation of Nuclear Extracts and STAT-1 Binding Assay.** Cytosolic and nuclear extracts were prepared from stimulated cells using a nuclear cytoplast extraction kit (Pierce Chemical, Rockford, IL). Protein concentration was assessed using the Bradford method.

Binding of STAT-1 was assessed on nuclear extracts using a kit for Lightshift Chemiluminescent EMSA following the protocol provided by the manufacturer (Pierce Biotechnology) with minor modifications. A double-stranded oligonucleotide containing a STAT-1 consensus sequence was labeled on the 3’ end with biotin. In brief, binding reaction mixtures containing 5 μg of nuclear protein, 10 mM Tris, 50 mM KCl, 1 mM dithiothreitol, 2.5% glycerol, 5 mM MgCl2, 0.05% Nonidet P-40, 50 ng of poly(dI·dC), and 40-fmol oligonucleotide probe were incubated for 20 min at room temperature. Specific binding was confirmed by using a 100- to 400-fold excess of unlabeled probe as specific competitor. Protein-DNA complexes were separated using 6% nondenaturing acrylamide gel electrophoresis. Complexes were transferred to positively charged nylon membranes and uncross-linked. Gel shifts were visualized with a streptavidin-horseradish peroxidase followed by chemiluminescent detection.

**RNA Interference of the STAT-1 Pathway.** To confirm that the increase in eosinophil adhesion to HBECysLT1R epithelial cells was causally linked to LTD4-dependent STAT-1 activation (Slezd and Williams, 2005), we tested the effect of p91/STAT-1 silencing, using specific siRNA transfection. Cells were plated in 24-well tissue culture plates and grown in medium containing 10% FBS without the use of antibiotic until 60 to 80% confluency. Later, p91/STAT-1 siRNA (10 μM; Santa Cruz Biotechnology, Inc.) was used to deliver 40 μl of siRNA transfection medium, and the reaction was performed according to the manufacturer’s instructions until complete transfection of cells (30 h at 37°C). Optimal siRNA transfection efficiency, control siRNA (10 μM; Santa Cruz Biotechnology, Inc.) was used containing a scrambled sequence that did not lead to the specific degradation of any known cellular mRNA. Finally, cells were stimulated with LTD4 for 18 h, and eosinophil adhesion was evaluated. The silencing efficacy of the RNA interference for p91/STAT-1α was checked by Western blot analysis.

**Statistical Analysis.** The data were expressed as median ± S.D. of the results of each experiment, unless otherwise stated. Eosinophil adhesion was analyzed using ANOVA test with Fisher’s test correction.

**Results**

LTD4 increased eosinophil adhesion to HBECysLT1R Cells, reaching a maximal response at concentrations ≥ 0.1 μM (Fig. 1A). As expected, the specific CysLT1R antagonist Montelukast (0.1–10 μM) inhibited in a concentration-dependent fashion the effect of LTD4 (0.1 μM) with a maximal response at concentrations ≥ 1 μM (Fig. 1B). The increase in eosinophil adhesion was accompanied by a statistically significant increase in the expression of ICAM-1 on the surface of HBECysLT1R cells (Fig. 2), as shown by fluorescence-activated cell sorting analysis of cells incubated with increasing concentrations of LTD4; as expected, also this effect was abolished by pretreating epithelial cells with the CysLT1R antagonist Montelukast (1 μM; data not shown).

The treatment of HBECysLT1R with LTD4 (0.1 μM) activated ERK1/2 in a time-dependent manner, as shown by the increase in the phosphorylated protein. Phospho-ERK1/2 was detectable by 5 min, maximal at 15 to 15 min, and returned to basal values after 30 min (Fig. 3). LTD4 did not seem to activate p38 MAPK, but the analysis of total protein lysates showed a significant activation of STAT-1 pathway, as shown by the time-dependent phosphorylation of STAT-1 in Ser727 in the presence of unchanged amounts of p91/STAT-1α. It is interesting to note that the stimulation of HBECysLT1R with LTD4 did not activate the phosphorylation of STAT-1 in Tyr701 (Fig. 4A, second lane).

As previously reported, treatment with IFN-γ (50 ng/ml) activated ERK1/2 phosphorylation, but at variance with the results observed with LTD4, significant phosphorylation of STAT-1 both in Ser727 and in Tyr701 was observed; the amounts of p91/STAT-1α were not increased by the incubation with IFN-γ (Fig. 4A). Costimulation of HBECysLT1R with IFN-γ (50 ng/ml) and LTD4 (0.1 μM) resulted in a
analysis of three independent experiments, carried out as described in Materials and Methods. Pretreatment with the bisindolylmaleimide PKC inhibitor GF109203X (Toullec et al., 1991), or the MEK inhibitor PD98059 prevented both the effects on signal transduction pathways associated with LTD₄-dependent cellular activation and the increase in eosinophil adhesion.

The inhibition of the nuclear translocation of p91/STAT-1α by Montelukast or by the MEK inhibitor observed in LTD₄-activated epithelial cells supported the hypothesis that the nuclear translocation of STAT-1 occurs through the CysLT₁R-dependent activation of the ERK1/2 pathway. Finally, silencing of STAT-1 protein expression in HBECsLT₁R cells completely suppressed the increase in eosinophil adhesion associated with challenge with LTD₄, providing evidence for the first time of a key contribution of STAT-1 activation to the activity of CysLTs in epithelial cells.

The airway epithelium represents a target as well as a source of inflammatory mediators and, through the expression of adhesion molecules, may contribute to the recruitment of inflammatory cells, ultimately leading to the pathophysiological changes typical of the asthmatic airways (Hamilton et al., 2001). IFN-γ may contribute to this phenomenon, increasing ICAM-1 expression (Look et al., 1992), a response typically associated with intracellular transduction signals leading to transcription and translation of specific genes (Look et al., 1992). Indeed, in airway epithelial cells, IFN-γ-inducible gene expression is associated with STAT-1-dependent pathway activation (Look et al., 1992). STAT-1 is selectively activated in airway epithelium of asthmatic subjects and correlates with ICAM-1 expression of airway epithelium of asthmatic subjects (Sampath et al., 1999), whereas the inhibition of the STAT-1 pathway attenuates airway inflammation and hyper-reactivity (Chen et al., 2004), suggesting that STAT-1 activation may play an important role in asthma (Quarcoo et al., 2004). The STAT-1 protein is a family of latent transcription factors that are acti-
vated by a wide range of cytokines. Upon engagement, STATs become tyrosine phosphorylated, translocate to the nucleus, and induce expression of target genes. At the same time, STAT-1 is phosphorylated on Ser727, independently of tyrosine phosphorylation (Wen et al., 1995), and although tyrosine phosphorylation is required for cytokine-induced STAT-1 dimerization, nuclear translocation, and DNA binding, full transcriptional activity of STAT-1 is substantially related to serine phosphorylation. Indeed, activation of STAT-1 through Ser727 phosphorylation independent from Tyr701 phosphorylation has been reported and may be involved in the induction of gene transcription regulating apoptosis and Fas receptor/Fas ligand expression (Stephanou et al., 2001). The mechanism of STAT-1 phosphorylation in Ser727 is not yet well understood, although the presence of a potential MAPK consensus PMSP motif suggests the involvement of ERK1/2 (Wen et al., 1995; Darnell,
Indeed, in addition to IFN-γ (Blanchette et al., 2003), several GPCRs, such as angiotensin II AT1 and thrombin receptors, can regulate STAT-1 activity through the interaction between ERK1/2 activity and STAT-1 serine phosphorylation (Schindler and Darnell, 1995).

It has been reported that CysLT1R mainly couples with pertussis toxin-insensitive G protein, although reports exist of coupling with Gαi in human cells (Capra et al., 2004, 2007). For the first time, we provide evidence that CysLT activation of HBECysLT1R epithelial cells causes activation of ERK1/2 leading to STAT-1 phosphorylation, nuclear translocation, ICAM-1 surface expression, and enhanced eosinophil adhesion; the significant inhibition of the CysLT activity observed using a selective PKC inhibitor (Toullec et al., 1991) also suggests the involvement of a PKC-dependent phosphorylation leading to the activation of Raf-1 (Hoshino et al., 1998; Paruchuri and Sjolander, 2003). Additional studies are necessary to fully elucidate the involvement of PKC in the transduction signaling of CysLT1R in epithelial cells.

ICAM-1 (CD54) binds to two integrins belonging to the β2 subfamily, CD11a/CD18 (leukotactic factor activity-1) and CD11b/CD18 (MAC-1), both expressed by leukocytes, resulting in adhesion and transendothelial migration of leukocytes from the bloodstream. Similar processes control leukocytes adhesion to lung airway epithelial cells and may contribute to the damage of these cells observed in asthma (Bloemen et al., 1997). Airway inflammation resulting from viral infection is indeed accompanied by marked leukocyte trafficking and production of CysLTs and IFN-γ (van Shaik et al., 2000). IFN-γ has also been reported to increase the expression of CysLT1R in myocytes (Amrani et al., 2001), to promote the release of CysLTs from human eosinophils (Saïto et al., 1988), and may have a role in the induction of airway hyper-responsiveness in ovalbumin-challenged experimental animals (Hessel et al., 1997).

Taken together, these evidence support a potential associa-

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**Fig. 6.** Effects of PKC and MEK inhibition on LTD4-induced eosinophil adhesion in epithelial cells. HBECysLT1R cells were stimulated with 0.1 μM LTD4 for 24 h in the presence or absence of GF109203X (10 μM, 30 min before epithelial cell stimulation) (A) or PD98059 (25 μM, 30 min before epithelial cell stimulation) (B). The adhesion of eosinophils was evaluated using fluorometric analysis as described under Materials and Methods. Results were expressed as median and S.D. of six independent experiments. Statistical analysis was performed using ANOVA test with Fisher's test correction. *, p < 0.05 versus untreated cells.

**Fig. 7.** STAT-1 binding to DNA in LTD4-treated epithelial cells. STAT-1 binding to DNA was evaluated by EMSA on nuclear extracts obtained from HBECysLT1R cells treated with LTD4 (0.1 μM, 30 min) in the presence or absence of Montelukast (1 μM, 1 h before epithelial cell stimulation) (A) or in the presence or absence of the specific MEK inhibitor PD98059 (25 μM, 30 min) (B). EMSA was performed with STAT-1 oligonucleotide probes as described under Materials and Methods.

**Fig. 8.** p91/STAT-1α silencing and LTD4-stimulated eosinophil adhesion to epithelial cells. Temporary transfection of HBECysLT1R cells with siRNA for p91/STAT-1α was carried out as described under Materials and Methods. Expression of p91/STAT-1α protein in control cells and cells transfected with scrambled siRNA or siRNA for p91/STAT-1α (A). Eosinophil adhesion to control cells and cells transfected with scrambled siRNA or siRNA for p91/STAT-1α in response to LTD4 treatment (0.1 μM, 24 h) (B). p91/STAT-1α was analyzed by Western blot using anti-p91/STAT-1α antibodies. Adhering cells were analyzed using fluorometric analysis as described under Materials and Methods. Results were expressed as median and S.D. of six independent experiments. Statistical analysis was performed by using ANOVA test with Fisher's test correction. *, p < 0.05 versus the respective untreated cells.
tion among ICAM expression, eosinophilic infiltration, CysLTs or IPN-γ production, and airway inflammation in response to antigen and during asthma exacerbation. Within this line of evidence, our data suggest that IFN-γ and LTD₄ may both contribute to the recruitment of eosinophils in the airway of asthmatic subjects via the activation of airway epithelium, leading to sustained bronchoconstriction and airway hyper-reactivity. Studies performed on the intestinal epithelial cells have demonstrated that LTD₄ regulates cell proliferation (Massoumi et al., 2002) via PKC-dependent stimulation of ERK1/2. Likewise, in airway smooth muscle cells, it has been shown that CysLT₁R activation induces PKC translocation (Accamozzo et al., 2001), as well as ERK1/2 activation through a Gₛ-dependent mechanism (Ravasi et al., 2006).

The results obtained in our studies, both using EMSA as well as STAT-1 silencing, for the first time provide evidence of the involvement of STAT-1 in the signal transduction mechanism associated with CysLT₁R activation, supporting the hypothesis that it may represent a key transduction pathway leading to the enhanced eosinophilic adhesiveness observed in response to CysLT activation. The inhibitory effect of Montelukast provides additional support to the potential anti-inflammatoory activity of CysLT₁R receptor antagonists.

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References


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