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Correspondence and requests for materials should be addressed to S.P. (sp97@uakron.edu)

Cysteinyl leukotrienes regulate endothelial cell inflammatory and proliferative signals through $CysLT_2$ and $CysLT_1$ receptors

Ernest Duah[1], Ravi K. Adapala[2], Nosayba Al-Azzam[1], Vinay Kondeti[1], Farai Gombedza[1], Charles K. Thodeti[2] & Sailaja Paruchuri[1]

[1]Department of Chemistry, University of Akron, OH 44325, [2]Integrative Medical Sciences, Northeast Ohio Medical University, Rootstown, OH 44272.

Cysteinyl leukotrienes (cys-LTs), LTC_4, LTD_4, LTE_4 are potent inflammatory lipid mediators that act through two distinct G-protein-coupled receptors, $CysLT_1R$ and $CysLT_2R$. Although cys-LTs are shown to induce vascular leakage and atherosclerosis, the molecular mechanism by which cys-LTs modulate endothelial function is not known. Here, we show that cys-LTs (LTC_4 and LTD_4) induce robust calcium influx in human umbilical vein endothelial cells (HUVECs) through $CysLT_2R$, but not $CysLT_1R$. Further, cys-LT treatment induced endothelial cell (EC) contraction leading to monolayer disruption via $CysLT_2R$/Rho kinase dependent pathway. Furthermore, stimulation with cys-LTs potentiated TNFα-induced VCAM-1 expression and leukocyte recruitment to ECs through $CysLT_2R$. In contrast, we found that both LTC_4 and LTD_4 stimulated EC proliferation through $CysLT_1R$. Taken together, these results suggest that cys-LTs induce endothelial inflammation and proliferation via $CysLT_2R$/Rho kinase and $CysLT_1R$/Erk dependent pathways, respectively, which play critical role in the etiology of cardiovascular diseases such as atherosclerosis and myocardial infarction.

Inflammation and endothelial dysfunction are the major contributors for the initiation and progression of atherosclerosis and its associated cardiovascular risks[1–3]. The initial activation of endothelium subsequently results in the production of pro-inflammatory molecules that interact with leukocytes and further propagates the inflammatory process leading to change in endothelial cell (EC) constitutive properties and abnormal state of the endothelium with compromised function[1–3]. Although research has shown that atherosclerosis is an inflammatory disease, there is incomplete understanding of the role of inflammatory lipid mediators in its pathogenesis. Leukotrienes are pro- inflammatory mediators generated from arachidonic acid cascade and have been implicated in atherosclerosis[3]. Cysteinyl leukotrienes (cys-LTs), comprising of LTC_4, LTD_4 and LTE_4 are implicated in inflammatory diseases such as asthma, rheumatoid arthritis and inflammatory bowel disease[3,4]. Cys-LTs exert their effects through two different G-protein-coupled receptors, $CysLT_1R$ and $CysLT_2R$[5,6]. Several cys-LT receptor antagonists have been approved by FDA and are in the market for the treatment of asthma and allergic rhinitis[7,8].

Although inflammatory cells were identified as the main source as well as target of cys-LTs, these lipids were also shown to be produced during vascular injury and affect vascular cell function. In the past, cys-LTs were shown to exert a broad variety of effects on cardiovascular system such as constriction of microvasculature, enhancement of permeability of post-capillary venules and reduction in coronary blood flow[9,10]. However, not much attention was given to cys-LTs in cardiovascular system until recently. The identification and characterization of G-protein coupled CysLTRs, $CysLT_1R$ and $CysLT_2R$ renewed the interest on cys-LTs. Specifically, $CysLT_2R$ has been recently shown to be involved in atherosclerosis and vascular leakage during myocardial injury and pathological retinal angiogenesis[11–14]. Since, cys-LTs are secreted by inflammatory cells in vascular wall during vascular injury it is conceivable that cys-LTs exert their effect on ECs. Endothelial $CysLT_2R$ overexpression was found to up-regulate the expression of genes including ICAM-1, and VCAM-1[15]. However, the molecular mechanisms by which cys-LTs regulate EC function are not known. Endothelial function is often de-regulated during atherosclerosis contributing to endothelial dysfunction which includes enhanced EC proliferation,

contraction of EC monolayer and increased permeability, expression of adhesion molecules and subsequent attachment of immune cells. In the current study, we analyzed the effects of cys-LTs on the modulation of above mentioned EC phenotypes as well as elucidate the mechanism of action behind.

Results

LTC_4 and LTD_4 induce calcium influx in HUVECs through $CysLT_2R$ but not $CysLT_1R$. In order to determine the role of cys-LTs in regulating endothelial function, first we measured the expression of their receptors, $CysLT_1R$ and $CysLT_2R$ in HUVECs. Quantitative RT-PCR analysis revealed that the expression of $CysLT_2R$ is higher in HUVECs compared to that of $CysLT_1R$ (Fig. 1A). Western blot analysis showed that these cells express both $CysLT_1$ and $CysLT_2$ receptors (Suppl Fig. 1). To determine the functional significance of these receptors, we measured cys-LT induced calcium flux in HUVECs loaded with Fluo-4 AM. We found that both LTC_4 and LTD_4 induced rapid calcium flux in these cells (Fig. 1B,C). Interestingly, cys-LT-induced calcium influx was significantly abolished in the presence of a specific $CysLT_2R$ antagonist[14], $BayCysLT_2$ (1 μM) (Fig. 1B,C). In contrast, a specific $CysLT_1R$ antagonist MK571 (1 μM) failed to inhibit calcium influx by either LTC_4 or LTD_4 (Fig. 1B, C). These results clearly suggest that cys-LTs induce calcium influx through the activation of $CysLT_2R$ in endothelial cells.

Leukotrienes induce endothelial contraction and endothelial barrier disruption through a Rho kinase-dependent mechanism. Cys-LTs were previously implicated in vascular leakage[14]. However, the molecular mechanism through which cys-LTs regulate EC contraction and permeability is not known. As a next step in understanding the role of cys-LTs in regulating EC function, we sought to determine if cys-LTs can induce contraction and barrier disruption in EC monolayer. EC were grown to a confluent monolayer, incubated with cys-LTs for 5 h followed by fixation and staining for F-actin. Treatment of monolayer with LTC_4 or LTD_4 induced EC contraction as evidenced by gap formation between the F-actin stained cells (Fig. 2A, B). In contrast, the EC monolayer was found intact in control non-treated cells. Thrombin stimulation induced robust gap formation in EC monolayer and served as a positive control. Quantitative analysis revealed that both LTC_4 and LTD_4 induced significant EC contraction compared to thrombin (80% and 90% of thrombin, respectively).

We then investigated which CysLTR was involved in the EC contraction. We found that LTD_4-induced EC contraction was significantly inhibited by $CysLT_2R$ antagonist, $BayCysLT_2$, but not $CysLT_1R$ antagonist, MK571 (Fig. 2C, Suppl Fig. 2). Rho kinase has been shown to modulate histamine and thrombin-induced barrier dysfunction[16]. To explore if Rho kinase has a role in mediating cys-LT-induced contraction in HUVECs, we employed a pharmacological Rho Kinase inhibitor, Y27632, in EC contraction assays. Pretreatment of cells with Rho kinase inhibitor, Y27632, significantly attenuated both LTC_4 and LTD_4-induced EC contraction and gap formation (Fig. 2C, Suppl Fig. 2).

Cys-LTs potentiate TNFα-induced responses in EC. Attachment of immune cells to endothelium, upon activation, is a distinct EC function which is a critical event in atherosclerosis. Hence, we explored if these potent pro-inflammatory cys-LTs can influence leukocyte attachment to EC. We found that LTC_4 or LTD_4 failed to induce expression of adhesion molecules or attachment of leukocytes to endothelium (Fig. 3). However, cys-LTs significantly

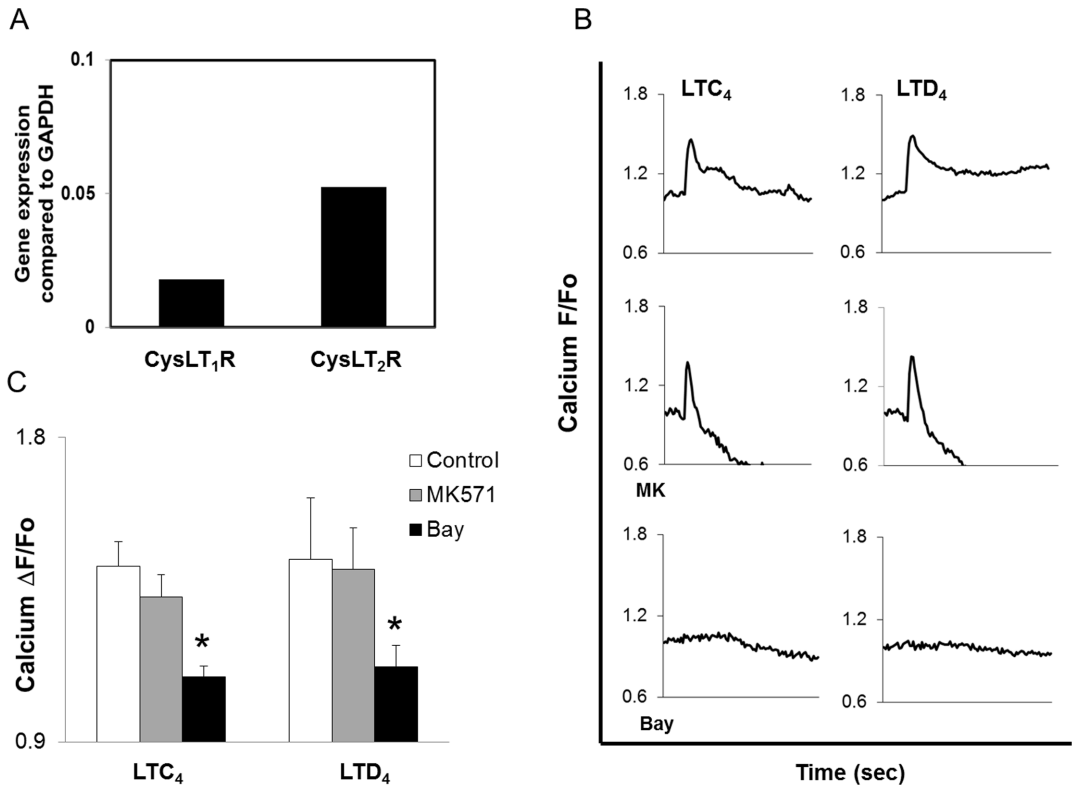

Figure 1 | Human endothelial cells (HUVECs) express both $CysLT_1R$ and $CysLT_2R$ and Cys-LTs induce calcium signaling through $CysLT_2R$ but not $CysLT_1R$. (A) RT-PCR analysis showing the expression of $CysLT_1R$ and $CysLT_2R$ in human endothelial cells. (B) HUVECs were loaded with Fluo-4 (4 μM) and stimulated with 500 nM of LTC_4 or LTD_4 and changes in fluorescence intensity was measured on confocal microscope in presence or absence of 1 μM MK571 or $BayCysLT_2$ (Bay). (B) Calcium transient showing cys-LT-induced calcium influx. (C) Quantitative analysis showing the Δ calcium changes. The results shown are mean ± SEM from 3 independent experiments. The significance was analyzed using student's t-test and set at $p \leq 0.05$.

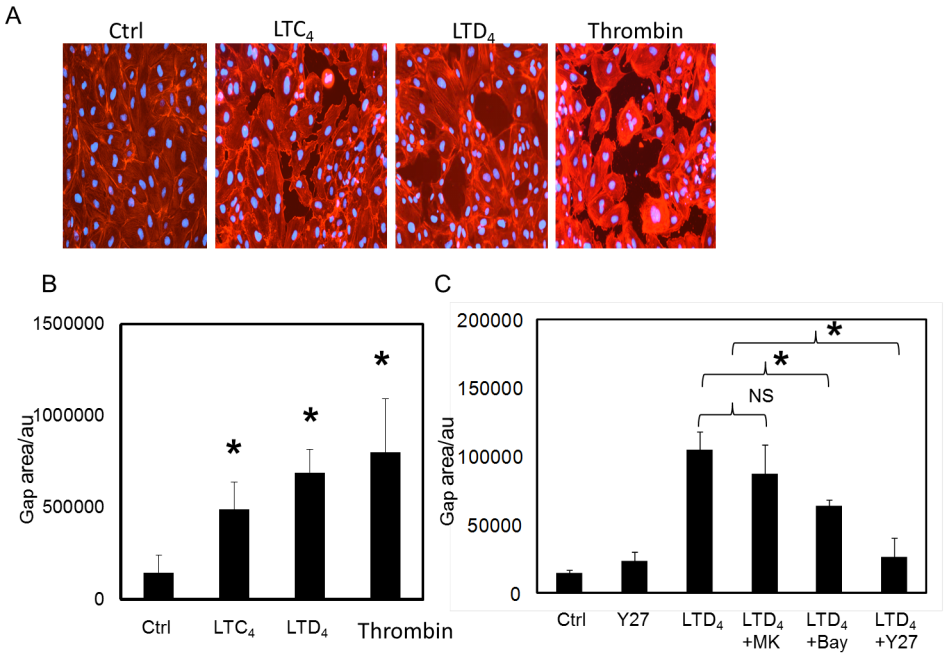

Figure 2 | cys-LTs induce EC contraction and gap formation via $CysLT_2R$/Rho kinase dependent pathway. Confluent monolayer (>95%) of HUVECs were serum starved overnight and treated for 5 h with 500 nM LTC_4 or LTD_4 or 0.1 U/mL Thrombin. Cells were stained for F-actin (Texas Red-X phalloidin) and nucleus (DAPI) and images were taken under fluorescence microscope. (A) Fluorescence micrographs showing increase in gap formation after treatment with 500 nM LTC_4 or LTD_4 or thrombin and as evidenced by F-actin (red) and nucleus (blue) staining. (B) Quantitative analysis showing the gap formation by LTC_4 and LTD_4 and (C) by LTD_4 in the presence and absence of a Rho kinase inhibitor, Y27632 (10 μM) or $CysLT_1R$ antagonist, MK571 (1 μM) or $CysLT_2R$ antagonist, $BayCysLT_2$ (Bay, 1 μM). The results shown are mean ± SEM from 3 independent experiments. The data was analyzed using one way ANOVA followed by Tukey post-hoc analysis. The significance was set at $p \leq 0.05$. NS = non significant.

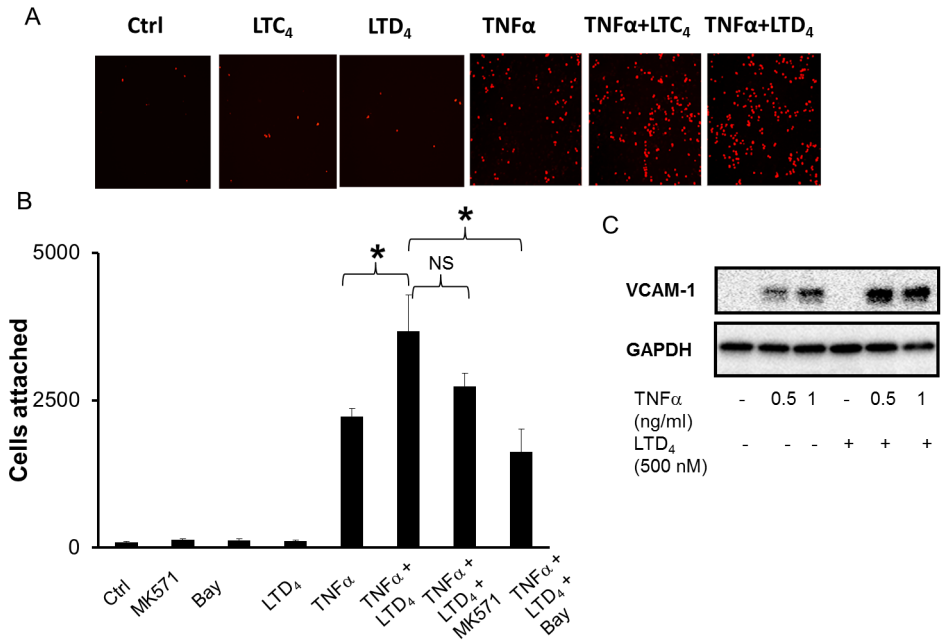

Figure 3 | LTC_4 and LTD_4 potentiate TNFα induced attachment of THP-1 cells to endothelial cells. Confluent HUVECs treated with 500 nM of LTC_4, LTD_4 or TNFα (0.25 ng/mL) for 4 h were incubated with THP-1 cells prior stained with $DiIC_{12}(3)$ fluorescent dye for 1 h. (A) Attachment of THP-1 cells to HUVECS after 5 times washing. Representative experiment is illustrated out of three performed. (B) Quantitative analysis showing the TNFα/LTD_4-induced attachment of THP-1 cells to EC monolayer in the presence or absence of MK571 or $BayCysLT_2$ (Bay). The results shown are mean ± SEM from 3 independent experiments. The data was analyzed using one way ANOVA followed by Tukey post-hoc analysis. The significance was set at $p \leq 0.05$. (C) A representative Western blot showing the expression of VCAM-1 by TNFα or LTD_4 alone or in combination. GAPDH served as a loading control.

potentiated expression of adhesion molecule VCAM-1 (Fig. 3C) and attachment of leukocytes in the presence of sub-physiological concentration of TNFα (Fig. 3A, B, Suppl Fig. 3). Notably, we found that this recruitment of leukocytes was significantly attenuated by $BayCysLT_2$, but not MK571 (Fig. 3A, B, Suppl Fig. 3). These findings suggest that cys-LTs potentiate inflammatory signals elicited by other distinguished inflammatory mediators such as TNFα and enhance recruitment of leukocytes to endothelium further steering the endothelial dysfunction via $CysLT_2R$.

Cys-LTs activate Erk and enhance proliferation through $CysLT_1R$ in HUVECs. We next asked whether cys-LTs regulate endothelial cell proliferation. First, we measured cell proliferation using XTT assay in serum starved HUVECs stimulated with cys-LTs. Both LTC_4 and LTD_4 stimulation resulted in significant increase in EC proliferation (Fig. 4A). To validate this result, we also measured proliferation by assessing BrdU incorporation into the cells by ELISA in response to LTD_4 (Fig. 4B). We found that LTD_4-induced EC proliferation was significantly inhibited by MK571, but not by $BayCysLT_2$, indicating a role for $CysLT_1R$ in endothelial proliferation (Fig. 4B). Basal as well as cys-LT-induced proliferation was completely blocked by PD98059, a MEK inhibitor (Fig. 4B) suggesting that proliferative signal in HUVECs is mediated through Erk. Along these lines, earlier reports also suggest that cys-LTs enhance cell proliferation through Erk in a number of cell types earlier[17,18]. This suggests that cys-LTs might induce cell proliferation via Erk phosphorylation in endothelial cells. In order to find out if ERK is the signaling intermediate in cys-LT-induced EC proliferation, we measured phosphorylation of Erk. As shown in Fig. 4C, LTD_4 significantly increased phosphorylation of Erk and this phosphorylation was transient, maximum at 5 min and declined.

Discussion

In the present study, we delineated the molecular mechanism by which cys-LTs regulate endothelial function and demonstrated that the cys-LTs induce inflammatory signals through $CysLT_2R$ and proliferation through $CysLT_1R$. We further demonstrate that $CysLT_2R$ activation results in EC contraction and barrier disruption through Rho kinase pathway and potentiate TNFα-induced attachment of leukocytes to endothelial monolayer via up-regulation of VCAM-1. To our knowledge, this is the first study to report the involvement of Rho kinase down-stream of $CysLT_2R$ in ECs. Finally, we demonstrated that EC proliferation is mediated through the activation of $CysLT_1R$.

Cys-LTs mediate their effects through the activation of $CysLT_1R$ and $CysLT_2R$ in different cell types[4,18–21]. $CysLT_2R$ was reported to be dominantly expressed in endothelial cells (HUVECs) and has been shown to mediate cys-LT-mediated calcium signaling[22]. Also, initial studies on HUVECs demonstrated that EC predominantly express $CysLT_2R$ and stimulation with LTD_4 induces early inflammatory genes[23] such as EGR (Early growth response) and NRS4A (nuclear receptor subfamily 4 group A) transcription factors, IL-8 (interleukin-8), DSCR1(Down syndrome critical region gene 1), E-selectin, CXCL2 (CXC ligand 2), ADAMTS1(a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif 1), TF (tissue factor) and COX2 (cyclooxygenase 2)[23]. Using pharmacological inhibitors, both $CysLT_1R$ and $CysLT_2R$ are implicated in

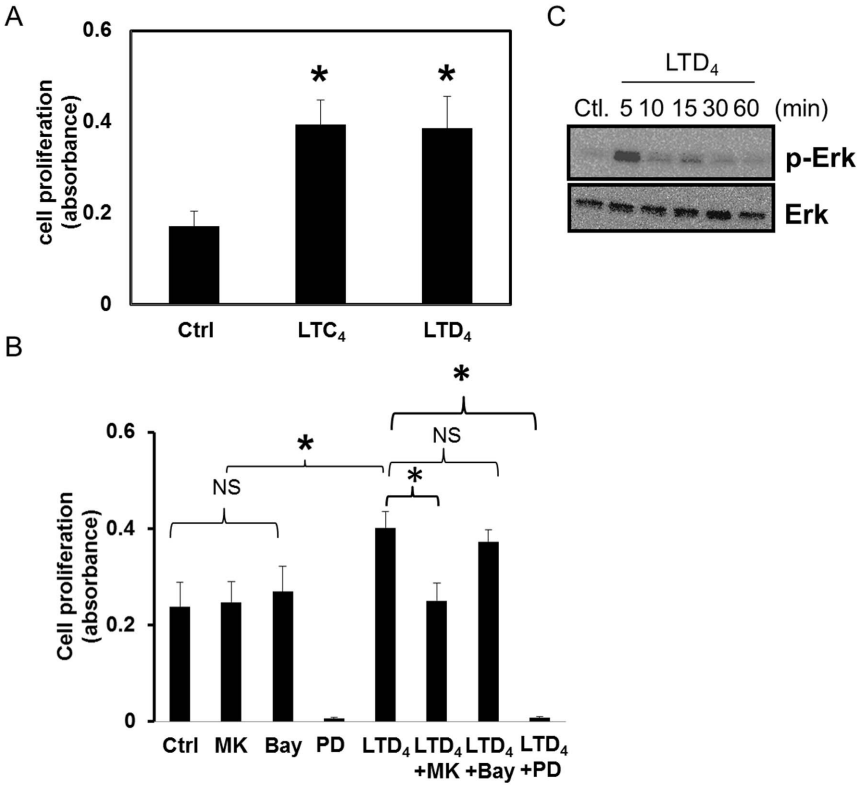

Figure 4 | Cys-LTs induce proliferation and Erk activation in HUVECs. (A) HUVECs were cultured in 96 well plate, serum starved overnight, then treated with or without 500 nM LTC_4 or LTD_4 for 48 h and proliferation was assayed by XTT assay. (B) HUVECs were cultured in 96 well plate, serum starved overnight, then treated with or without 500 nM LTD_4 for 48 h in the presence or absence of 1 μM of MK571 or $BayCysLT_2$ (Bay) or PD98059 (50 μM). BrdU was added during the last 24 h and its incorporation into the cells was assayed by ELISA. The results shown are mean ± SEM from 2 independent experiments. The data was analyzed using one way ANOVA followed by Tukey post-hoc analysis. The significance was set at $p \leq 0.05$. (C) HUVECs were stimulated with 500 nM of LTD_4 for indicated points of time, lysed, proteins were separated and Erk phosphorylation was measured using phospho-specific antibodies. Total Erk was used as a loading control.

vascular permeability and ischemia-induced cerebral, renal and myocardial injury[13,24–26]. $CysLT_1R$ expression appears to be up-regulated in renal endothelial cells and correlated with ischemia-reperfusion injury in rat kidney[24]. Interestingly, $CysLT_1R$ was shown to translocate to nucleus in response to oxygen-glucose deprivation-induced damage in brain endothelial cells[27]. On the other hand, $CysLT_2R$ was demonstrated to play critical role in vascular leakage during myocardial injury and pathological retinal angiogenesis[13,25,26]. This vascular permeability seems to be mediated through trans-endothelial vesicle transport regulated by $CysLT_2R$-induced calcium signaling[26]. Despite the clear demonstration that CysLTRs are critical mediators of vascular leakage, the molecular mechanism by which cys-LTs/CysLTRs mediate these effects are not known.

The present study identified three important mechanisms of cys-LT signaling that include a) Rho kinase as a down-stream regulator of $CysLT_2R$-induced endothelial contraction b) potentiation of TNFα-induced leukocyte recruitment by $CysLT_2R$ signaling through the up-regulation of VCAM-1 expression, and c) modulation of EC proliferation by $CysLT_1R$. Notably, these mechanisms provide molecular basis for leukotriene-induced inflammatory EC phenotype in atherosclerosis as well as ischemia-induced vascular injury. Previous studies demonstrated that LTD_4 induces expression of early inflammatory genes via activation of $CysLT_2R$ in HUVECs that are implicated in atherosclerosis. Interestingly, a well-known pro-thrombotic substance, thrombin also induced expression of similar genes in endothelial cells[23]. Moreover, LTD_4 together with thrombin increased the fold expression of these genes suggesting that cys-LTs act in concert with other mediators and also activate similar signaling mechanism[23]. The observations in the present study that both cys-LTs induce EC contraction and gap formation through Rho kinase coupled with the fact that thrombin induces EC permeability through Rho kinase[28,29] support the notion that both thrombin and LTD_4 activate similar mechanisms, although they stimulate different G-proteins. Our study also presents an evidence that cys-LTs potentiate effects of an inflammatory cytokine, TNFα on endothelial cells via increased expression of VCAM-1 and leukocyte recruitment through $CysLT_2R$. Thus, our results provide strong evidence for a role for $CysLT_2R$ signaling in the mediation of inflammatory EC phenotype.

Interestingly, calcium influx which is an immediate response of GPCR activation is exclusively mediated by $CysLT_2R$ but not $CysLT_1R$ in ECs. On the other hand, $CysLT_1R$ seems to be the preferred receptor for calcium influx in epithelial and inflammatory cells[18,30]. Calcium can activate a plethora of signaling events that modulate proliferation, migration, contraction and gene expression[31]. Although we do not know the exact role calcium plays in cys-LT-induced signaling in EC, previous studies have demonstrated that it acts up-stream of $CysLT_2R$-induced trans-endothelial vesicle transport and vascular leakage[26].

In addition to $CysLT_2R$ signaling, our study demonstrates that cys-LTs mediate EC proliferation through the activation of $CysLT_1R$. We also found that cys-LTs activate Erk in endothelial cells. In contrast, LTD_4 failed to induce proliferation in EA.hy926 (a HUVEC line), but promoted migration via $CysLT_1R$ mediated Erk activation[32]. Previous studies have shown that LTD_4 induce proliferation of intestinal epithelial cells[17,20] and mast cells[18,21] through $CysLT_1R$ but has no direct effect on fibroblast proliferation[33] suggesting cell specific effects which may explain the difference in proliferation in HUVECs and EA.hy926 cell line. Although we did not measure migration, LTD_4 was shown to mediate migration in intestinal epithelial cells through $CysLT_1R$[34]. Taken, together these findings suggest that cys-LTs regulate endothelial function via both $CysLT_1R$ and $CysLT_2R$. However, it is not known how and whether particular CysLTR is activated by cys-LTs.

In conclusion, our study presents the molecular mechanism for the regulation of endothelial phenotype by cys-LTs and provides evidence for the activation of both $CysLT_1R$ and $CysLT_2R$ which induce proliferative signals via probable activation of Erk and inflammatory signals via Rho kinase and VCAM-1 expression. Our study shows the possible involvement of these inflammatory signals in two important pathological conditions a) Rho kinase-dependent EC contraction and increased gap formation which increases EC permeability in ischemia-induced vascular leakage and b) potentiation of TNFα-mediated increased expression of VCAM-1 and leukocyte recruitment which are critical events in the initiation of atherosclerosis. Understanding these down-stream molecular mechanisms of cys-LTs in the regulation of endothelial function may provide new therapeutic targets for the treatment of cardiovascular diseases such as ischemic heart disease, oxygen-induced retinopathy and atherosclerosis.

Methods

Materials. LTC_4, LTD_4, MK571, $BayCysLT_2$, $CysLT_1R$ and $CysLT_2R$ antibodies were from Cayman Chemicals, Ann Arbor, MI. Validated real time primers for $CysLT_1R$ and $CysLT_2R$ were purchased from SABiosciences (cat # PPH02507A and PPH15153A). Phospho and total Erk antibodies were from cell signaling technology (Danvers, MA). VCAM-1 (E-10) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All secondary antibodies were obtained from Jackson Immunoresearch laboratories (West Groove, PA). XTT proliferation assay kit was from Trevigen (Gaithersburg, MD) and BrdU proliferation assay kit was purchased from CalBiochem EMD Millipore (La Jolla, CA). Human TNFα was obtained from Peprotech, Inc. (Rocky Hill, NJ). Thrombin and Y27632 were obtained from Sigma (St. Louis, MO) and Tocris Bioscience (Mineapolis, MN), respectively. Fluo-4 AM and Texas Red-X phalloidin were purchased from Molecular Probes (Eugene, OR) and $DilC_{12}$ (3) fluorescent dye was purchased from BD Bioscience (Bedford, MA).

Cell culture. Human umbilical vascular endothelial cells (HUVECs) were maintained in EBM2 medium with EGM2 Bullet supplements, 10% fetal bovine serum and maintained at 37°C in a humidified 5% CO_2 environment.

Calcium imaging. HUVECs cultured on glass bottomed dishes (MatTek) were loaded with Fluo-4/AM (1–4 μM) for 30 min and washed in calcium medium (136 mM NaCl, 4.7 mM KCl,1.2 mM $MgSO_4$, 1.1 mM $CaCl_2$, 1.2 mM KH_2PO_4, 5 mM $NaHCO_3$, 5.5 mM glucose, and 20 mM Hepes. pH 7.4). Cells were stimulated with LTC_4 or LTD_4 (500 nM) in calcium medium in the presence or absence of CysLTR antagonists, MK571 (1 μM) and $BayCysLT_2$ (1 μM) pre-incubated for 30 min. Calcium imaging was performed on Olympus Fluo View 300 confocal microscope and analyzed using Fluo View software and Microsoft Excel[30,35–38].

Endothelial monolayer contraction, F-actin staining and microscopy. ECs were grown on cell culture plates, serum starved and stimulated with cys-LTs (LTC_4 or LTD_4; 500 nM) or thrombin (0.1 U/ml) for 5 h, rinsed with phosphate-buffered saline (PBS) and fixed for 20 min at room temperature in PBS containing 4% paraformaldehyde. Following fixation, cells were permeabilized with PBS containing 0.25% Triton-X100, blocked with BSA and incubated with Texas Red conjugated phalloidin to stain actin stress fibers. Images were obtained using EVOS fluorescence microscope using 20× objective. Images were processed using Image J (NIH) software. In some cases, cells were pre-treated with specified antagonists for 30 minutes before stimulation with the indicated agonists.

Attachment assay. ECs were cultured as described above, serum starved and stimulated with or without cys-LTs (LTC_4 or LTD_4; 500 nM) and or TNFα 0.25 ng/mL for 4 hr. In some cases, cells were pre-treated with specified antagonists before stimulation with the indicated agonists. After stimulations, cells were washed with RPMI medium and co-incubated with THP-1 cells for 1 hr at 4°C prior stained with $DilC_{12}$(3) fluorescent dye as described by the manufacturer protocol. Unattached cells were washed 5 times with PBS and attached cells counted under a fluorescent microscope. Data were analyzed using NIH Image J and Microsoft Excel.

Cell lysates and Western blotting. After stimulation with the respective agonists, ECs were lysed with lysis buffer (BD Bioscience) supplemented with protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Pierce). In some experiments, cells were pre-treated for 30 min with respective antagonists as specified. All the antagonists used in the current study were dissolved in DMSO. Control cells were stimulated with DMSO alone. In some experiments, cells were also treated with inhibitors alone without agonist treatment. Immuno-blotting was performed as described previously[17]. Briefly, lysates were subjected to 4–12% SDS-PAGE and transferred to PVDF membrane. Membranes were incubated with respective primary phospho- and total antibodies diluted in 1× TBS, 5% dry milk, 0.1% Tween-20 (1 : 1000) overnight at 4°C on shaker, and then with secondary antibody (peroxidase-conjugated anti-rabbit or anti-mouse). Western blot was incubated with ECL and the bands were visualized using imager (Protein Simple) and quantified using Image J (NIH).

Real-time quantitative PCR. The expressions of $CysLT_1R$, $CysLT_2R$ transcripts were determined with real-time PCR performed on Light cycler 480 (Roche). HUVECs were cultured as described above and total RNA was isolated with an RNAeasy minikit (Qiagen) and cDNA was synthesized using cDNA synthesis kit from Quanta Biosciences containing qScript Reverse Transcriptase. Real time PCR was performed using real time $CysLT_1R$, $CysLT_2R$ and GAPDH Primers purchased from Superarray and SYBR green PCR master mix from Quanta Biosciences. The levels of $CysLT_1R$ and $CysLT_2R$ relative to the GAPDH levels was analyzed and the ΔΔCT values are expressed as fold change.

Cell proliferation. HUVECs were plated at the density of 1500 cells/well of 96 well plate, serum starved overnight and were treated with 500 nM of LTC_4 and LTD_4. After 48 h, the proliferation was assayed by XTT or BrdU ELISA according to the manufacturer's protocols. BrdU label was added 24 h before the assay. In some experiments, cells were pre-incubated for 30 minutes with 1 μM of MK571 or $BayCysLT_2$ or 50 μM of PD98059.

Data analysis. All the data shown is mean ± SEM from at least three independent experiments. Significance was determined using Student's *t* test as well as one-way ANOVA followed by Tukey post-hoc analysis and was set at $p < 0.05$.

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Author contributions

E.D., R.A., N.A., V.K. and F.G. performed experiments and analyzed the data. C.K.T. provided cells, designed some of the experiments, and edited the manuscript. S.P. designed, performed research, interpreted, analyzed data and wrote the manuscript.

Additional information

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