Cysteinyl leukotriene 2 receptor and protease-activated receptor 1 activate strongly correlated early genes in human endothelial cells


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Cysteinyl leukotrienes (cysLT), i.e., LTC4, LTD4, and LTE4, are lipid mediators derived from the 5-lipoxygenase pathway, and the cysLT receptors cysLT1-R and cysLT2-R mediate inflammatory tissue reactions. Although endothelial cells (ECs) predominantly express cysLT1-Rs, their role in vascular biology remains to be fully understood. To delineate cysLT2-R actions, we stimulated human umbilical vein EC with LTD4 and determined early induced genes. We also compared LTD4 effects with those induced by thrombin that binds to protease-activated receptor (PAR)-1. Stringent filters yielded 37 cysLT2-R- and 34 PAR-1-up-regulated genes (>2.5-fold stimulation). Most LTD4-regulated genes were also induced by thrombin. Moreover, LTD4 plus thrombin augmented gene expression when compared with each agonist alone. Strongly induced genes were studied in detail: Early growth response (EGR) and nuclear receptor subfamily 4 group A transcription factors; E-selectin; CXC ligand 2; IL-8; a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif 1 (ADAMTS1); Down syndrome critical region gene 1 (DSCR1); tissue factor (TF); and cyclooxygenase 2. Transcripts peaked at ~60 min, were unaffected by a cysLT1-R antagonist, and were superinduced by cycloheximide. The EC phenotype was markedly altered: LTD4 induced de novo synthesis of EGR1 protein and EGR1 localized in the nucleus; LTD4 up-regulated IL-8 formation and secretion; and LTD4 raised TF protein and TF-dependent EC procoagulant activity. These data show that cysLT2-R activation results in a proinflammatory EC phenotype. Because LTD4 and thrombin are likely to be formed concomitantly in vivo, cysLT2-R and PAR-1 may cooperate to augment vascular injury.

Cysteinyl leukotriene 2 receptor gene signature | protease-activated receptor 1 gene signature | vascular inflammation

Leukotrienes (LTs), i.e., LTE4 and the cysteinyl LTs (cysLT) LTC4, LTD4, and LTE4 constitute a group of lipid mediators derived from the 5-lipoxygenase (5-LO) pathway (1, 2). LTs are either produced by leukocytes at sites of inflammation or formed through transcellular metabolism after uptake and metabolism of leukocyte-derived LTs by downstream enzymes of the 5-LO pathway (LTA4 hydrolase and LTC4 synthase) in cells that normally do not express 5-LO, such as endothelial cells (ECs) (3, 4). LTs act through G protein-coupled surface receptors (GPCRs), i.e., the LTB4 receptors and the cysLT LT receptors (LT-Rs) (cysLT1-R and cysLT2-R) (5–10). LT-Rs are expressed on multiple target cells, including leukocytes, smooth muscle cells, and ECs (1). Recent studies implicate the 5-LO pathway in cardiovascular disease (11–17).

Considerable information is available on cysLT1-R, whereas little is known about cysLT2-R. We have used human umbilical vein (HUVECs) as a model of vascular cells to study cysLT2-R activation by demonstrating that cysLTs exclusively signal through cysLT2-R in this cell type (18). In fact, HUVECs are the first primary cell type that selectively expresses cysLT2-R. In the study detailed below, we determined HUVEC gene signatures in response to LTD4 and characterized the resulting phenotypes. LTD4 responses were compared with those of the prototype vasoactive agonist thrombin, which, in HUVEC, acts through protease-activated receptor (PAR)-1 (19). Thrombin was used as a positive control for LTD4 because it shares many acute effects with LTD4 on ECs, both act through GPCRs, and thrombin and LTD4 may be formed concomitantly during vascular injury (see below). Our data show that cysLT2-R activation induces early gene signatures that resemble those after PAR-1 activation and that the combined actions of LTD4 and thrombin further stimulate gene expression. Thus, if LTD4 and thrombin are produced at the same time and site, they may cooperate in EC activation during states of vascular perturbation in vivo.

**Results**

Early LTD4-Induced Genes Resemble Those Induced by Thrombin. HUVECs were stimulated with 100 nM LTD4 or 10 nM thrombin for 60 min in cultures from four umbilical cords, and microarrays were analyzed [Gene Expression Omnibus (GEO) accession no. GSE3589]. To identify regulated genes, stringent filter criteria were applied. LTD4 induced 37 genes >2.5-fold at 60 min (Fig. 1 A and B; and see Supporting Text and Table 1, which are published as supporting information on the PNAS web site). LTD4-induced genes were the result of cysLT2-R activation, because quantitative (Q)RT-PCR analyses showed barely detectable cysLT1-R transcripts but significant numbers of cysLT2-R transcripts, and induction of all 13 genes studied was resistant toward a specific cysLT1-R antagonist (See Fig. 6, which is published as supporting information on the PNAS web site, and data not shown). In addition, similar to Ca2+ responses (18), LTC4 and LTD4 were equipotent in gene activation (data not shown) and cysLT2-R transcripts exceeded cysLT1-R transcripts by a factor of 1,000 (see Table 2, which is published as supporting information on the PNAS web site). We next compared LTD4 and thrombin responses in parallel cultures using 10 nM thrombo-

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Abbreviations: ADAMTS1, a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif 1; COX, cyclooxygenase; cysLT, cysteinyl leukotriene; DSCR1, Down syndrome critical region gene 1; EC, endothelial cell; EGR, early growth response; LO, lipoxygenase; LT, leukotriene; LTD4, LT-R; LT receptor; GPCR, G protein-coupled receptor; HUVEC, human umbilical vein EC; NFAT, nuclear factor of activated T cells; NR4A, nuclear receptor subfamily 4 group A; SELE, E-selectin; QRT-PCR, quantitative RT-PCR; TF, tissue factor; PAR, protease-activated receptor.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database (accession no. GSE3589).

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bin. Thrombin induced 34 genes >2.5-fold at 60 min (Fig. 1A and C). Most of the 37 LTD4-induced genes were also significantly induced by thrombin if not, in few instances, by a factor of >2.5-fold (Fig. 1A). Thus, when LTD4-regulated early genes were examined regarding their induction by thrombin, a significant correlation was apparent (Pearson correlation coefficient, r = 0.90). These data revealed that activation of cysLT2-R generates early gene signatures that resemble those after PAR-1 stimulation by thrombin (Fig. 1A–D; and see Tables 3–6, which are published as supporting information on the PNAS web site).

**Fig. 1.** Early LTD4- and thrombin-regulated genes in HUVECs. HUVECs were stimulated with 100 nM LTD4 or 10 nM thrombin. (A) Heatmap of genes up-regulated >2.5-fold by LTD4; thrombin was used for comparison. (B and C) Scatterplots of LTD4- and thrombin-stimulated cells versus control; lines depict 2.5-fold change. (D) Scatterplot of LTD4- versus thrombin-stimulated cells. (E and F) Comparison of gene expression of LTD4-plus-thrombin-treated cells with cells stimulated with LTD4 or thrombin alone. Probe sets were selected as described in Supporting Text. Line separates up- from down-regulated probe sets. Columns in A indicate umbilical cord preparations; dots in scatterplots indicate means of signal intensities of four (B–D) or three (E and F) umbilical cords.
In addition to the 60-min time point, genes at 6 and 24 h after stimulation with LTD₄ were determined in a single array experiment (see Tables 7–14, which are published as supporting information on the PNAS web site). We next focused on genes that were up-regulated at 60 min.

**LTD₄ and Thrombin Cooperate to Enhance Gene Expression.** LTD₄ and thrombin act through distinct members of GPCR subfamilies, cysLT₂-R and PAR-1 (5–10, 19–22), yet they share many acute effects on ECs in vitro and in vivo and may be formed concomitantly during vascular injury in vivo (see Discussion). We examined whether concomitant activation of cysLT₂-R and PAR-1 by addition of LTD₄ plus thrombin enhances gene expression when compared with each agonist alone. For this purpose, gene signatures of HUVECs prepared from three umbilical cords were determined in response to LTD₄ plus thrombin parallel to LTD₄ or thrombin alone (see Tables 15 and 16, which are published as supporting information on the PNAS web site). Fifty genes were up-regulated by at least one of the two agonists, and their overall expression was significantly augmented by the concomitant addition of both agonists when compared with each agonist alone (Fig. 1 A and Table 1) as well as cyclooxygenase (COX)-2. To ascertain that array signal intensities represented up-regulated transcripts, induction of 13 genes (including COX-2; data not shown) was confirmed by QRT-PCR analyses at 1 h, and transcript kinetics were determined (Figs. 2–5; and see Fig. 7, which is published as supporting information on the PNAS web site; and data not shown). Transcript levels of all genes peaked at ~60 min after addition of LTD₄. Because many of them are immediate-early genes in other agonist-response systems (23), we examined their sensitivity toward protein-synthesis inhibition. Each of the LTD₄-induced genes examined (n = 13) was superinduced in the presence of the protein-synthesis inhibitor cycloheximide (Fig. 3 Inset and data not shown). These data show that many, if not all, of the LTD₄-regulated genes at the 60-min time point (Fig. 1 A) belong to the immediate-early gene family (23).

**CysLT₂-R Activation Generates a Comprehensive Immediate-Early Gene Program.** LTD₄-regulated genes were found to encode transcription factors, signaling molecules, an inhibitor of calcineurin signaling, chemokines, angiogenic factors, extracellular matrix-degrading molecules, adhesion molecules, and tissue factor (TF) (Fig. 1 A and Table 1) as well as cyclooxygenase (COX)-2. To ascertain that array signal intensities represented up-regulated transcripts, induction of 13 genes (including COX-2; data not shown) was confirmed by QRT-PCR analyses at 1 h, and transcript kinetics were determined (Figs. 2–5; and see Fig. 7, which is published as supporting information on the PNAS web site; and data not shown). Transcript levels of all genes peaked at ~60 min after addition of LTD₄. Because many of them are immediate-early genes in other agonist-response systems (23), we examined their sensitivity toward protein-synthesis inhibition. Each of the LTD₄-induced genes examined (n = 13) was superinduced in the presence of the protein-synthesis inhibitor cycloheximide (Fig. 3 Inset and data not shown). These data show that many, if not all, of the LTD₄-regulated genes at the 60-min time point (Fig. 1 A) belong to the immediate-early gene family (23).

**LTD₄ Stimulates Early Growth Response (EGR) and Nuclear Receptor Subfamily 4 Group A (NR4A) Transcription Factors.** LTD₄ stimulated transcripts of 14 transcription factors (Fig. 1 A). The EGR and...
NR4A families were chosen for detailed analyses because they may contribute to inflammation. EGR1 can induce TF by transactivating the TF promoter, and EGR1 has been shown to mediate oxidative stress-induced vascular reperfusion injury (24–27). Members of the EGR and NR4A families were among the most strongly up-regulated genes, as judged by the fold change of signal intensities of the corresponding probe sets (Table 1). QRT-PCR analyses showed that transcripts of each transcription factor reached different absolute expression levels (Figs. 2A and 3). Transcript levels of EGR1 and -3 differed by a factor of 10, that of EGR1 and -2 by a factor of >10 (Fig. 2A), and EGR4 was not induced. EGR1 transcript up-regulation was followed by a pronounced induction of EGR1 protein that became visible at 1 h, appeared as a protein doublet thereafter, and had returned to near baseline levels at 4 h (Fig. 2B). Immunolocalization experiments showed that EGR1 was present in the nucleus at 1 h in LTD4-stimulated cells (Fig. 2C). The magnitude of LTD4’s action on EGR1 was comparable to that of TNFα (Fig. 2 B and C). Of NR4A family members, NR4A1 was the most strongly affected member that was induced to an ~5-fold-higher level than that of NR4A2 or -3 (Fig. 3). These data show that LTD4 markedly stimulates transcription factors implicated in EC activation and reperfusion injury and that EGR1 translocates to the nucleus subsequent to its LTD4-induced de novo synthesis.

LTD4 Stimulates Expression of Genes Implicated in EC Activation and Two Antinflammatory Genes. We chose additional genes implicated in EC-activation regulation for detailed analyses, i.e., E-selectin (SELE), a disintegrin-like and metalloprotease with thrombospondin type 1 motif 1 (ADAMTS1), Down syndrome critical region gene 1 (DSCR1), CXCL2 (MIP2), and CXCL8 (IL-8) (28–32), SELE, ADAMTS1, and DSCR1 transcripts peaked ~60 min and then declined but remained elevated for 6 h (Fig. 7). By contrast, elevated transcript levels of CXCL2 and IL-8 had returned to near baseline levels at 4 h (Figs. 4 and 7). Up-regulation of IL-8 transcripts was followed by IL-8 secretion into the culture medium, with a maximum at 2 h (Fig. 4 Inset). Furthermore, two LTD4-responsive genes have established antiinflammatory actions, i.e., COX-2 (data not shown) and DSCR1 (Fig. 7): COX-2 is a key prostacyclin-synthesis-regulating enzyme in ECs (33), and DSCR1 has been shown to strongly down-regulate proinflammatory calcineurin/nuclear factor of activated T cells (NFAT)-transactivated genes by directly inhibiting calcineurin (31, 34–36). In preliminary studies, we observed marked up-regulation of COX-2 protein and prostacyclin formation (K.L. and A.J.R.H., unpublished work). Moreover, the strong LTD4-triggered DSCR1 (target of calcineurin in HUVECs) transcript up-regulation (Fig. 7) was markedly attenuated by the immunosuppressive agent cyclosporin (data not shown). These data provide preliminary evidence that the Gαi-coupled Ca2+/calcineurin/NFAT pathway may be involved in gene activation of HUVECs by cysLT2-R signaling.

LTD4 Up-Regulates TF- and HUVEC-Associated Procoagulant Activity. TF is the key regulator of the extrinsic blood coagulation cascade and has been shown to play multiple roles in vascular biology (37). Up-regulation of TF transcripts by LTD4 peaked at ~60 min and then declined but revealed biphasic kinetics with a second peak at 2–3 h, and elevated TF transcripts were maintained for ~4 h (Fig. 5A), followed by induction of TF protein with a maximum at 6 h (Fig. 5B). Large differences of TF antigen concentrations and TF-dependent procoagulant activities were recently noticed in blood (38, 39). To explore whether the LTD4-mediated increase in TF antigen was accompanied by concomitant EC-associated TF-dependent procoagulant activity, formation of active Factor Xa after formation of the TF/Factor VIIa complex was determined (see Materials and Methods). LTD4 induced cell-associated procoagulant activity by a factor of >4-fold (Fig. 5C).

Discussion

These studies support three major conclusions: (i) The diverse nature of LTD4-induced early genes suggests that cysLT2-R signaling gives rise to a comprehensive early proinflammatory and prothrombotic EC phenotype and to two antiinflammatory genes; (ii) simultaneous LTD4/cysLT2-R and thrombin/PAR-1 signaling enhances early gene expression when compared with each agonist alone; and (iii) early LTD4- and thrombin-induced gene signatures are similar. Because LTD4 and thrombin may be formed concomitantly during vascular injury (3, 4, 20, 22, 39), our data are consistent with the possibility that two rather distinct GPCR subfamily members (5–10, 20–22, 40–42), cysLT2-R and PAR-1, cooperate to promote EC activation in vivo.
Binding of LTD₄ to cysLT₂-R activates genes that have not previously been associated with the action of cysLTs. These include transcription factors that participate in ischemic stress and reperfusion injury in mice (24–27) (EGRI and NR4A); chemokines and cell-adhesion molecules involved in blood leukocyte recruitment to inflamed tissues (28–30, 43) (CXCL2, IL-8, and SELE); an extracellular matrix-degrading metalloprotease implicated in carotid artery remodeling (44) and degradation of the collagen-binding proteoglycans versican and aggrecan (32) (ADAMTS1), a potent endogenous inhibitor of calcineurin signaling (31) (DSCR1), the key regulator of the extrinsic blood coagulation cascade (42) (TF), and a key prostaglandin synthesis-regulating enzyme (33) (COX-2). These data show that cysLTs initiate activities through robust induction of proinflammatory genes. These gene activations follow previously noticed activities of LTD₄ that occur within seconds as a direct result of cysLT₂-R signaling (1–4, 7, 11, 14, 16, 18, 40, 41, 45), and they also indicate that cysLT₂-R signaling activates at least two genes that may limit inflammation and, therefore, may be involved in attenuation of the otherwise potent proinflammatory actions of cysLTs (COX-2 and DSCR1). Moreover, cysLT₂-R may signal through the Ca²⁺/calmodulin/calcineurin/ NFAT pathway, as evidenced by the potent inhibitory action of the immunosuppressant, i.e., cyclosporin, on LTD₂-mediated DSCR1 transcript up-regulation that we observed in three independent experiments (data not shown). It is noteworthy that DSCR1 may provide a major endogenous feedback inhibitor of NFAT signaling of inflammation in T cells and HUVECs by other GPCR and non-GPCR activation events (31, 34–36). Several genes up-regulated in response to cysLT₂-R activation and studied here in detail are up-regulated in HUVECs by other GPCRs, such as the major HUVEC thrombin receptor (PAR-1) or the histamine receptor (46) in a cyclosporin-sensitive manner (31, 34, 46). Thus, several of the early genes may be the result of Gα protein coupling, but additional G proteins may also be involved (6). The observation that the cysLT₂-R in human mast cells was shown to up-regulate IL-8 in a Ca²⁺-insensitive manner (47) indicates, however, that cysLT₂-R may couple G proteins in a cell-type-specific way (Gα versus Gβγ). Alternatively, cysLT₂-R may couple several G proteins similar to other GPCRs. In recombinant systems, cysLT₂-R and thrombin receptors appear to couple predominantly through Gα-mediated phospholipase C β-triggered Ca²⁺ elevation, but coupling through Gγ has also been reported for thrombin in ECs (6, 48). That Gγ may participate in cysLT₂-R signaling in HUVECs was indicated by a partial inhibition of LTD₄-dependent ERK1/2 phosphorylation by pertussis toxin (K.L. and A.J.R.H., unpublished work). More work is required to identify HUVEC cysLT₂-R-coupling of G proteins, downstream signaling components, and the precise role of NFAT-regulated gene activation. The potent LTD₄ induction of the EGR1 gene and the subsequent nuclear localization of EGR1 protein deserves special attention, because EGR1 transactivates secondary genes in response to ischemia and reperfusion injury in mice (26), whereas high levels of EGR1-inducible genes have been observed in mouse and human atherosclerosis (25). We are presently attempting to identify cysLT₂-R-dependent secondary genes in response to the immediate-early genes identified here, although the TF promoter and other gene promoters have already been shown to be transactived by EGR1 (27). Identification of these receptor-dependent genes may also uncover differences between thrombin/PAR-1- and LTD₄/cysLT₂-R-dependent modulation of EC activation, because these GPCRs may conceivably serve distinct biological roles in vivo.

The similarities in LTD₄’s and thrombin’s immediate-early gene signatures (Fig. 1) add a new dimension to previously noticed shared responses of LTD₄ and thrombin. The latter include reduction of blood pressure, initiation of platelet-activating factor synthesis, secretion of von Willebrand factor, and surface expression of P-selectin, all of which occur within seconds and can be assumed to result from acute cysLT₂-R signaling independent of gene transcription (11, 42). By contrast, elaboration of immediate-early genes by a single LTD₄ and/or thrombin challenge renders HUVECs to an apparent activated phenotype that may persist for hours, if not longer (42). However, these proinflammatory activities may be attenuated by COX-2 and DSCR1 up-regulation, as noted above. That the cysLT₂-R mediates subacute types of fibroproliferative lung injury in vivo in response to bleomycin has recently been reported in cysLT₂-R-deficient mice (40, 41). Our data are consistent with the possibility that biological activities of the 5-LO pathway may result in lasting alterations of the EC phenotype, such as increased platelet–EC interactions, promotion of blood monocyte adhesion to the endothelium, participation in extracellular matrix remodeling, and reperfusion injury of the heart (40, 41). The shared activities of LTD₄ and thrombin invite examination of the possibility that still further actions of thrombin in vivo may also be relevant for LTD₄ actions on EC cysLT₂-R (20, 42). It is likely that thrombin and LTD₄ are formed concomitantly (3, 4, 20, 42). It is tempting to speculate that cysLTs may participate in the formation of complex inflammatory circuits (14) in diseased blood vessels through cysLT₂-R-mediated inflammatory EC gene targeting. This finding poses the interesting suggestion that cysLT₂-R-specific antagonists may be of benefit in cardiovascular disease.

Materials and Methods

Materials. LTD₄ was obtained from Cayman Chemical (Hamburg, Germany), TNFα from R & D Systems (Wiesbaden-Nordenstadt, Germany), and thrombin and all other reagents from Sigma-Aldrich (Munich), unless otherwise noted; anti-EGRI was from Santa Cruz Biotechnology, Cy3-conjugated donkey anti-rabbit antibody from DianoVa (Hamburg, Germany), and anti-TF antibody from American Diagnostica (Pfungstadt, Germany). Montelukast was a gift of R. N. Young, Merck Frosst (Quebec, Canada). Reagents for QRT-PCR analyses were from Invitrogen (Karsruhe, Germany).

Microarray Analysis. RNA was extracted and purified by TRizol and RNeasy Micro kit (Qiagen, Hilden, Germany). RNA integrity was determined on RNA 6000 Nano LabChips (Agilent Technologies, Palo Alto, CA). Preparation of cRNA and hybridization on U133A arrays were performed according to the manufacturer’s protocols (Affymetrix, Santa Clara, CA). Additional information is published in Supporting Text. Microarray data of LTD₄- and thrombin-stimulated HUVECs are published in National Center for Biotechnology Information’s Gene Expression Omnibus at www.ncbi.nlm.nih.gov/geo (GEO accession no. GSE3589).

Cell Culture and ELISA of IL-8. HUVECs were maintained in serum-free medium (Invitrogen) and used within the first two passages. Cells were stimulated with 100 nM LTD₄, 10 nM thrombin, and 100 units/ml TNFα or solvent for varying time periods. For IL-8 measurements, the medium, including LTD₄, was replaced, supernatants were collected, and IL-8 was determined by ELISA (BD Biosciences, Heidelberg).

QRT-PCR. RNA was reverse-transcribed, and external standards were prepared and sequence confirmed as described in ref. 16. QRT-PCR parameters were established by using SYBR green as a detection dye as described in ref. 16, with primers reported in Table 18, which is published as supporting information on the PNAS web site.

Western Blot. Cells were lysed for analysis of EGR1 in Laemmli buffer or for TF in buffer containing 10 mM Tris-HCl, pH 7.5,
10 mM MgCl₂, 1% Triton X-100, and the protease-inhibitor mixture Complete (Roche Diagnostics, Mannheim, Germany). Proteins were separated by 10% SDS/PAGE and blotted onto nitrocellulose membrane (GE Healthcare, Freiburg, Germany). Membranes were blocked and incubated with EGR1 (1:6,000) or TF- (1:4,000) specific antisera. Immunoreactive bands were detected by chemiluminescence and CDP-Star reagent (Applied Biosystems, Darmstadt, Germany).

Immunofluorescence. HUVECs were cultured on coverslips (Nunc, Wiesbaden, Germany). After stimulation, the cells were fixed with 4% formaldehyde and permeabilized with 0.5% Triton X-100. The cover slips were incubated with anti-EGR1 (1:100) and, after washing, with the secondary antibody (Cy3-conjugated anti-rabbit antibody, 1:600). DNA was stained with DAPI. Confocal laser scanning microscopy was performed with a Zeiss Axiovert 200 microscope equipped with a LSM 510 laser scanner head.


TF-Dependent Procoagulant Activity. Cells were lysed in a buffer of 50 mM Tris-HCl, pH = 7.4, 100 mM NaCl, and 0.1% Triton X-100, sonicated, and assayed by using a TF chromogenic activity assay according to the manufacturer’s protocol (American Diagnostic, Greenwich, CT).

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