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IFN-γ Induces Cysteinyl Leukotriene Receptor 2 Expression and Enhances the Responsiveness of Human Endothelial Cells to Cysteinyl Leukotrienes

Grzegorz Woszczek,* Li-Yuan Chen,* Sahrudaya Nagineni,* Sara Alsaaty,* Anya Harry,* Carolea Logun,* Rafal Pawliczak,*† and James H. Shelhamer2*

Cysteinyl leukotrienes (cysLTs) are important mediators of cell trafficking and innate immune responses, involved in the pathogenesis of inflammatory processes, i.e., atherosclerosis, pulmonary fibrosis, and bronchial asthma. The aim of this study was to examine the regulation of cysLT signaling by IFN-γ in human primary endothelial cells. IFN-γ increased cysLT receptor 2 (CysLTR2) mRNA expression and CysLTR2-specific calcium signaling in endothelial cells. IFN-γ signaled through Jak2/STAT1, as both AG490, a Jak2 inhibitor, and expression of a STAT1 dominant-negative construct, significantly inhibited CysLTR2 mRNA expression in response to IFN-γ. To determine mechanisms of IFN-γ-induced CysLTR2 expression, the human CysLTR2 gene structure was characterized. The CysLTR2 gene has a TATA-less promoter, with multiple transcription start sites. It consists of six variably spliced exons. Eight different CysLTR2 transcripts were identified in endothelial and monocytic cells. Gene reporter assay showed potent basal promoter activity of a putative CysLTR2 promoter region. However, there were no significant changes in gene reporter and mRNA t1/2 assays in response to IFN-γ, suggesting transcriptional control of CysLTR2 mRNA up-regulation by IFN-γ response motifs localized outside of the cloned CysLTR2 promoter region. Stimulation of endothelial cells by cysLTs induced mRNA and protein expression of early growth response genes 1, 2, and 3 and cyclooxygenase-2. This response was mediated by CysLTR2 coupled to Gα11s, activation of phospholipase C, and inositol-1,4,5-triphosphate, and was enhanced further 2- to 5-fold by IFN-γ stimulation. Thus, IFN-γ induces CysLTR2 expression and enhances cysLT-induced inflammatory responses. 


1 Abbreviations used in this paper: cysLT, cysteinyl leukotriene; 5-LO, 5-lipooxygenase; 5’UTR, 5’ untranslated region; Cox, cyclooxygenase; CysLTR, cysLT receptor; Egr, early growth response; EST, expressed sequenced tag; GPCR, G protein-coupled receptor; HMVEC-L, human microvascular endothelial cells from the lung; IP3, inositol-1,4,5-triphosphate; LTC4, leukotriene C4; LTD4, leukotriene D4; PLCβ, phospholipase Cβ; TSS, transcription start site.

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1 The nucleotide sequences presented in this article have been submitted to GenBank with the following accession numbers: EF141523, EF141524, EF141525, EF141526, EF141527, EF141528, EF141529, and EF141530.

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examined the regulation of CysLTR expression and their proinflammatory functions by IFN-γ in human endothelial cells.

Materials and Methods

Materials

LTC4, LTD4, MK571, and polyclonal anti-cyclooxygenase (Cox)-2 Ab (Cayman Chemical); BAYu9773 and pertussis toxin (Gq inhibitor) (BIOMOL); 2APB (inositol-1,4,5-triphosphate (IP3) inhibitor) and U73122 (phospholipase Cβ (PLCβ) inhibitor) (EMD Biosciences); human IFN-γ (R&D Systems); AG490, actinomycin D, and anti-β-actin Ab (Sigma-Aldrich); anti-early growth response (Egr) genes 1, 2, and 3 Abs (Santa Cruz Biotechnology); and peroxidase-conjugated anti-goat and anti-rabbit Abs (Jackson ImmunoResearch Laboratories) were obtained from the manufacturers. Wild-type STAT1 and mutant STAT1 dominant-negative expression plasmids were provided by M. Holtzman (Washington University School of Medicine, St. Louis, MO) (19).

Cell culture

HUVECs (Cambrex) were cultured in EBM-2 medium supplemented with EGM-2 (Cambrex) and used within the first or the second passage. Cells from at least two different donors were used in experiments. Human microvascular endothelial cells from the lung (HMVEC-L) (Cambrex) were grown in EBM-2 medium supplemented with EGM-2 MV (Cambrex) and used between passages 3 and 4. Human elutriated monocytes were obtained from the National Institutes of Health Blood Bank. THP-1 (acute monocytic leukemia) and U937 (human histiocytic lymphoma) (American Type Culture Collection) cell lines were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and 2 mmol/L L-glutamine (Invitrogen Life Technologies).

Real-time PCR

Total RNA was extracted from HUVECs and HMVEC-L using QIA Shredder columns and RNaseA kits; treated with DNase (Qiagen); and quantitated using a NanoDrop spectrophotometer (BioLabNet). mRNA expression for selected genes was measured using real-time PCR performed on an ABI Prism 7900 sequence detection system (Applied Biosystems) using the following commercially available probe and primers sets (Applied Biosystems): CysLTR1, Hs00272624_s1; CysLTR2, Hs00525658_s1; Egr1, Hs00152928_m1; Egr2, Hs00166165_m1; Egr3, Hs00231780_m1; and Egr4, Hs00231095_m1. For Cox-2 mRNA expression, the following primers and probe were used: forward primer, 5'-GCTCAACACTATGATGTTGGCATTC-3'; reverse primer, 5'-GCTGGCCCTCGCTTATGA-3'; and probe, TGCCCGACCTTACGCAGCTATGA. Reverse transcription and PCR were performed with a RT kit and TaqMan Universal PCR master mix (Applied Biosystems), according to manufacturer’s directions. Relative gene expression was normalized to GAPDH transcripts and calculated as a fold change compared with control. The relative (number of analyzed gene transcripts) for 1000 GAPDH transcripts baseline mRNA expression levels were as follows: CysLTR2, 2.44; Egr1, 44.8; Egr2, 4.8; Egr3, 4.25; and Cox-2, 39.1. For CysLTR2 mRNA expression, experiments, HUVECs were stimulated with IFN-γ (10 ng/ml) for 36 h; transcription was stopped by actinomycin D (5 μg/ml); and CysLTR2 mRNA was measured by TaqMan in control and stimulated cells.

Calcium mobilization assay

Calcium mobilization experiments were conducted using a FLIPR Calcium 3 assay kit (Molecular Devices), according to the manufacturer’s instructions. HUVECs (50,000 cells/well) were plated into 96-well plates 24 h before assay. The growth medium was removed and replaced with EGM-2 supplemented with 10 mmol/L HEPES and FLIPR 3 assay reagent. After incubation for 1 h at 37°C, fluorescence was measured every 4 s for 2 min using the FlexStation (Molecular Devices). The cells were pretreated for 10 min with MK571 (100 mmol/L), BAYu9773 (100 mmol/L), or vehicle, and stimulated with LTC4 or LTD4 (100 nmol/L). The growth medium was removed and replaced with EGM-2 supplemented with 10% heat-inactivated FBS and 2 mmol/L L-glutamine (Invitrogen Life Technologies), and sequenced using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Plasmid construction

Human CysLTR2 promoter fragments were cloned into pGL3-basic plasmid reporter vector (Promega) using the method previously described (20). A set of fragments up to 3012 bp long (numbers refer to the length of cloned promoter fragments with 5’ end localized to position +1 in relation to 3’ end of exon 1 as nt +1) of the identified CysLTR2 putative promoter region was amplified from human genomic DNA (Stratagene) by PCR with the following primers containing consensus sequences for KpnI and SacI restriction enzymes: p3012 F1, 5'-GACTAGTGGTGGTACCTACAGATCCAGAAAGCAAGCGATGAGG and R1, 5'-GACTAGTGGTGGTACCTACCTATCCCTCTCTGTCCCTTC; p1305 F2, 5'-GACTAGTGGTGGTACCTACCTATCCCTCTCTGTCCCTTC; and p976 F3, 5'-GACTAGTGGTGGTGGTACCTACCTATCCCTCTCTGTCCCTTC. The following CysLTR2 gene-specific primers were used: GSP1, 5'-CCAGGAAAGCAAGCGACACACTGCAGCAG and GSP2 nested, 5'-GATAGGGAGATGGATGGTTGCACAGG; GSP3, 5'-GACTAGTGGTGGTACCTACCTATCCCTCTCTGTCCCTTC and GSP4 nested, 5'-GACTAGTGGTGGTACCTACCTATCCCTCTCTGTCCCTTC. The nested PCR products were gel purified, cloned into pCR4-TOPO vector (Invitrogen Life Technologies).
The 3' deletion constructs were made by removing the indicated number of nucleotides from the 3' end of the insert in the p976 construct using the following primers: p976, 3'-36 F3 and R2, 5'-GACTAGGTCGAGCTCAGAAGGGAGCTTG; p976, 3'-96 F3 and R3, 5'-GACTAGGTCGA GCTCTGACCTGCTACACTTCC. PCR products were gel purified, cut with restriction enzymes, and ligated into pGL3-basic using LigAfast DNA ligation system (Promega). All plasmids were extracted using the EndoFree Plasmid Maxi kit (Qiagen), quantitated, and sequenced.

FIGURE 2. Calcium mobilization responses to cysLTs. HUVECs were prepared and calcium release was measured, as indicated in Materials and Methods. Cells were exposed or not to IFN-γ (10 ng/ml) for 48 h and stimulated with LTC₄ (100 nmol/L) (A) or LTD₄ (100 nmol/L) (B–D). For inhibition experiments, cells were preincubated with MK571 (100 nmol/L), BAYu9773 (100 nmol/L), or vehicle control (Con) (ethanol) for 10 min. Data from one of three experiments, each with similar results, are shown. E, LTD₄ induced dose response of control and IFN-γ (10 ng/ml, 48 h)-stimulated HUVECs. Data (mean ± SD) from two separate experiments done in triplicates are presented.
Transfections and luciferase gene reporter assay
HUVECs and HMVEC-L were cotransfected with 1/μg of the luciferase deletion construct and 0.05/μg of pRL-SV40 control vector using Lipofectin (Invitrogen Life Technologies) in serum-free medium (Opti-MEM I; Invitrogen Life Technologies) and harvested 48 h later. Dual-luciferase reporter assay (Promega) was performed using a Victor 1420 counter (PerkinElmer). Firefly luciferase activity was normalized to Renilla activity to account for transfection efficiency. In IFN-γ-stimulated experiments, cells were transfected and exposed to IFN-γ (10 ng/ml) for 12–72 h before harvesting.

HUVECs were transfected with STAT1 plasmids and control pcDNA plasmid, as above, and stimulated with IFN-γ (10 ng/ml) for 48 h; CysLTR2 mRNA was measured by real-time PCR.

Immunoblotting
Cells were collected into a buffer containing 50 mmol/L HEPES, 0.25% Triton X-100, and Complete Mini protease inhibitor mixture (Roche) and sonicated. Proteins (25 μg) were separated on 10% SDS Tris-glycine gel (Invitrogen Life Technologies), transferred to membranes, blocked with ECL Advance blocking agent, and incubated with primary Abs overnight at 4°C (1/1000 dilution), followed by the appropriate peroxidase-conjugated secondary Abs for 1 h at room temperature. The membranes were developed using an ECL Advance Western Blotting Detection kit (Amer sham Biosciences) and analyzed using the Image Station 440 (Eastman Kodak). For control β-actin, Abs were stripped from membranes using Blot Fresh Stripping Reagent (SignaGen Laboratories) and developed as above.

Statistical analysis
Data were analyzed by one-way ANOVA or paired and unpaired Student’s t tests, as appropriate. Differences were considered significant when p < 0.05.

Results
IFN-γ up-regulates CysLTR2 expression
The effect of IFN-γ on CysLTR expression was evaluated in cultured primary endothelial cells. As shown in Fig. 1, IFN-γ treatment increased the expression of CysLTR2 mRNA in a time- and dose-dependent fashion in human endothelial cells, with the greatest increase observed between 24 and 48 h. The expression of CysLTR2 mRNA increased in control cells as they became more confluent (Fig. 1) and decreased with the passage number (data not shown). CysLTR1 mRNA was expressed at or below the detection limits for quantitative PCR and was not changed by IFN-γ stimulation in either cell type. To determine whether the increased CysLTR2 mRNA translates into enhanced signaling by CysLTR2, intracellular calcium mobilization in response to cysLTs was examined. LTC4 and LTD4 (100 nmol/L) triggered similar calcium release in nonstimulated HUVECs (Fig. 2, A and B). This signaling was not inhibited by a selective CysLTR1 inhibitor MK571 and was partially inhibited by preincubation with BAYu9773 (Fig. 2C), which is a partial agonist for CysLTR2. Stimulation with IFN-γ (10 ng/ml) for 48 h caused an increase in calcium flux that was not inhibited by MK571 and was partially inhibited by BAYu9773 preincubation (Fig. 2, A and B). BAYu9773 alone acted on HUVECs as a weak agonist, and the calcium response to BAYu9773 was also increased by IFN-γ (data not shown). LTD4 induced calcium flux in a concentration-dependent fashion in control and IFN-γ-treated cells (Fig. 2E). Thus, IFN-γ treatment

![FIGURE 3. Structure of the human CysLTR2 gene. A, Identified exons are shown as boxes and numbered in relation to genomic contig AL137118. ATG indicates the translation start codon for CysLTR2. B, A fragment of exon 1 is shown with identified TSS in 5'RACE experiments (numbers refer to the 3' end of exon 1 as nt 1). C, Identified splice variants of CysLTR2 gene. A cross indicates cells in which particular transcripts were found.](http://www.jimmunol.org/10.4049/jimmunol.2001055)
To elucidate a mechanism of IFN-γ/H9253 activity CysLTR2 characterization of antagonist MK571, and partial inhibition by BAYu9773 preincubated cells. Seventeen different transcription start sites (TSS) were localized to position (numbers refer to the length of cloned promoter fragments with 3′ end deletion of 36 and 96 bases). Results are presented as fold values over an empty pGL3-basic vector. The means ± SD from three separate experiments each done in triplicate are shown.

Induced CysLTR2 mRNA and substantially augmented cysLT signaling in endothelial cells. The equal potency of LTC4 and LTD4, the lack of inhibition of cysLT signaling by the separate experiments each done in triplicate are shown.

FIGURE 4. Luciferase gene reporter activity in HUVECs (A) and HMVEC-L (B) transfected with CysLTR2 promoter deletion constructs (numbers refer to the length of cloned promoter fragments with 3′ end localized to position −43 in relation to 3′ end of exon 1 as nt −1; 3′−36 and 3′−96 indicate 3′ deletion of 36 and 96 bases). Results are presented as fold values over an empty pGL3-basic vector. The means ± SD from three separate experiments each done in triplicate are shown.

Characterization of CysLTR2 gene structure and promoter activity

To elucidate a mechanism of IFN-γ-induced up-regulation of CysLTR2 expression, the CysLTR2 gene structure was determined. The 5′RACE was performed on total RNA extracted from HUVECs, HMVEC-L, human monocytes, U937, and THP-1 cell lines. All obtained sequences matched to a genomic contig AL137118, and after alignment, six major exons were identified (Fig. 3A). Two variants of exon 2 were found that were named exons 2 and 2′, respectively. Five exons are localized to the 5′ untranslated region (5′UTR), and exon 6 contains the full coding region without interrupting intronic sequences. All intron/exon junctions followed the canonical GT-AG rule. Eight different transcripts of CysLTR2 were found (Fig. 3C); all of them contained the same exons 1 and 6. In HUVECs, transcript III was the most abundant, representing ~90% of detected sequences. Similarly, after IFN-γ-stimulation, transcript III was the only one detected, suggesting that this variant is the major CysLTR2 transcript in HUVECs. We were unable to detect CysLTR2 transcripts by 5′RACE in nonstimulated HMVEC-L, but in IFN-γ-stimulated cells transcripts VII and VIII were found with similar frequency. All other splice variants were detected in human monocytes or monocytic cell lines, suggesting that alternative splicing of CysLTR2 may be regulated at the cell- or tissue-type specific level. There was no common transcript predominance in IFN-γ-stimulated cells. Seventeen different transcription start sites (TSS) were found (Fig. 3B), all in the first exon. They were grouped into a proximal region between nt −53 and −92 (numbers refer to the last nucleotide at the 3′ end of exon 1 as number −1) and a distal one between −235 and −289. All transcripts detected in endothelial cells originated from the proximal region, mainly from TSS in position −89. Similarly, TSS −89 was the most common TSS in IFN-γ-stimulated endothelial cells. In monocytes and monocytic cell lines, CysLTR2 transcripts started in both regions, with predominance of transcripts originating in the proximal region.

FIGURE 5. Activation of Jak2/STAT1 is required for IFN-γ-mediated CysLTR2 induction. A. IFN-γ stimulation does not change CysLTR2 mRNA stability. HUVECs were cultured and treated, as indicated in Materials and Methods. Data (mean ± SD) are presented as percentage of control and IFN-γ-treated samples at time 0 (actinomycin D added) from two experiments, each done in triplicate. B. AG490 inhibits IFN-γ-induced CysLTR2 mRNA expression. HUVECs were stimulated with IFN-γ (10 ng/ml) or vehicle for 48 h, and CysLTR2 mRNA was measured by TaqMan. Cells were pre-exposed to different concentrations of AG490 or vehicle control (ethanol) for 30 min before IFN-γ stimulation. Data are presented as fold change over control vehicle-treated cells. Mean ± SD; n = 6–9; *, in comparison with IFN-γ-treated cells, p < 0.001, ANOVA. C. A STAT1 dominant-negative vector (Stat1 DN) inhibits IFN-γ-induced CysLTR2 mRNA expression. HUVECs were transfected, as indicated in Materials and Methods, and stimulated with IFN-γ (10 ng/ml) for 48 h. Results are shown as fold increase from control non-IFN-γ-treated cells. Mean ± SD; n = 9; *, in comparison with pcDNA-transfected and IFN-γ-stimulated cells, p < 0.02.
BLAST search of GenBank human mRNA sequences and expressed sequenced tags (EST) revealed five mRNA and seven EST sequences, all matching fully or partially some of the CysLTR2 transcripts identified by us.

A luciferase gene reporter assay was used to define a CysLTR2 promoter activity. Deletion constructs were made covering a region between positions -43 and -3055 of the first exon and promoter region, and promoter activities were measured (Fig. 4). In both transfected HUVECs and HMVEC-L, the putative region promoting basal transcription was localized in the region between nt -168 and -1350, with a construct p224 showing the highest activity. Deletion of the 3' end of the insert, which removed the proximal TSS, destroyed promoter activity as well.

Activation of Jak/STAT1 is required for IFN-γ-mediated CysLTR2 induction

To determine the mechanism of IFN-γ induction of CysLTR2 expression, HUVECs and HMVEC-L were transfected with luciferase deletion constructs and stimulated with IFN-γ, and gene reporter activity was measured. There was no significant difference in luciferase activity between control and IFN-γ-stimulated cells for all CysLTR2 constructs made (data not shown). To assess whether stimulation with IFN-γ affected CysLTR2 mRNA stability, HUVECs were treated with IFN-γ, and after 36 h transcription was inhibited by actinomycin D and CysLTR2 mRNA decay was measured by TaqMan. There was no significant difference in CysLTR2 mRNA half-life between control and IFN-γ-stimulated cells (Fig. 5A). IFN-γ-dependent gene activation is strongly related to activation of Jak and STAT1 proteins. In HUVECs, pretreatment with the Jak2 inhibitor AG490 30 min before IFN-γ stimulation significantly inhibited IFN-γ-mediated CysLTR2 mRNA induction, whereas vehicle-treated control and AG490 alone had no effect (Fig. 5B). To further define the IFN-γ-mediated pathway, HUVECs were transiently transfected with a dominant-negative STAT1 (Tyr701 mutated), wild-type STAT1, and control empty vectors, and after an overnight incubation stimulated with IFN-γ. The IFN-γ mediated CysLTR2 mRNA increase was significantly inhibited only in the dominant-negative STAT1-transfected cells (Fig. 5C).

IFN-γ stimulation enhances cysLT-induced Egr and Cox-2 expression

To determine whether IFN-γ up-regulation of CysLTR2 expression has functional consequences for endothelial cell function, Egr1, Egr2, Egr3, Egr4, and Cox-2 mRNA and protein expression was
studied in HUVECs. In nonstimulated cells, LTC4 induced an increase in Egr1, Egr2, Egr3, and Cox-2 mRNA expression, with maximum response observed after 60 min of stimulation, followed by similar changes at the protein level (Fig. 6, A and C). IFN-γ stimulation caused a significant (2- to 5-fold) increase in LTC4-mediated Egr1, Egr2, Egr3, and Cox-2 mRNA expression (Fig. 6B). Similar changes were observed at the protein level, with a maximum level of expression extended from 1 to 2 h after stimulation with LTC4 (Fig. 6C). This effect was dose dependent, it was not inhibited by MK571, and it was partially inhibited by BAYu9773 preincubation (Fig. 6D). IFN-γ alone had no significant effect on mRNA and protein expression of the genes studied. Egr4 mRNA was below the detection limit in control cells and was not changed by IFN-γ and LTC4 stimulation.

CysLTs induce Egr expression through CysLTR2 coupled to a Gαq/11/PLCβ/IP3 pathway
To determine the CysLTR2 signaling pathway for cysLT-induced Egr expression, HUVECs were pretreated with the Gαq inhibitor, pertussis toxin (100 ng/ml); the PLCβ inhibitor, U73122 (5 μmol/L); and the IP3 inhibitor, 2APB (100 μmol/L), before stimulation with LTC4 (100 nmol/L) for 1 h. Egr3 mRNA was assayed by TaqMan. Data are presented as fold change of Egr3 mRNA in comparison with vehicle-treated control. Mean ± SD, n = 6; * p < 0.001; ** p < 0.001 in comparison with LTC4-treated cells by Student’s t test.

![FIGURE 7. CysLTs induce Egr3 expression through CysLTR2 coupled to a Gαq/11/PLCβ/IP3 pathway. HUVECs were treated with pertussis toxin (PT) (100 ng/ml) for 24 h, with the PLCβ inhibitor U73122 (5 μmol/L) for 5 min and IP3 inhibitor 2APB (100 μmol/L) for 5 min before stimulation with LTC4 (100 nmol/L) for 1 h. Egr3 mRNA was assayed by TaqMan.](http://www.jimmunol.org/)

Discussion
We describe in this study three major findings, as follows: 1) a significant role of IFN-γ and the Jak/STAT1 pathway in up-regulation of CysLTR2 expression and its proinflammatory functions in human endothelial cells; 2) characterization of the structure and alternative splicing of the human CysLTR2 gene; and 3) partial determination of the CysLTR2 signaling pathway leading to Egr gene up-regulation. IFN-γ is a pleiotropic cytokine playing an important role in immune responses. We showed in this study that IFN-γ stimulation induces expression of CysLTR2 in endothelial cells, and, in addition, that this increased expression translates into enhanced signaling through release of intracellular calcium and enhanced expression of target genes, such as Egr1, Egr2, Egr3, and Cox-2. IFN-γ signals through the heterodimeric IFN-γ receptor, resulting in oligomerization, phosphorylation, and activation of Jak1 and Jak2. Subsequent Jak-mediated phosphorylation of the IFN-γ receptor leads to STAT1 phosphorylation on tyrosine 701, STAT1 dimerization, and nuclear translocation (21). Our data from HUVECs support this classical pathway of IFN-γ signaling operating in the case of CysLTR2 induction, because both AG490, which is a Jak2 inhibitor, as well as expression of a STAT1 dominant-negative (Tyr701I22I22I22 mutated) significantly inhibited CysLTR2 mRNA expression in response to IFN-γ. To determine whether IFN-γ-mediated increased expression of CysLTR2 is regulated by the CysLTR2 promoter at the transcriptional level, we characterized the CysLTR2 gene structure. The results are based on RACE experiments with a modified method that ensures the amplification of only full-length transcripts via elimination of truncated messages from the amplification process by removing 5′ phosphates. The human CysLTR2 gene consists of six exons that are variably spliced. Eight alternative transcripts were identified in endothelial cells, monocytes, and mononocytic cell lines. Although the pattern of transcripts detected in studied cell types was different (i.e., between endothelial cells and monocytes), suggesting cell-specific CysLTR2 alternative splicing regulation, we cannot exclude the possibility that alternative transcripts are generated in more random fashion. It seems likely that IFN-γ stimulation does not affect significantly alternative transcript selection because no dominant transcript was detected after IFN-γ exposure. However, most of the transcripts started at position −89 of the first exon after IFN-γ stimulation, suggesting that this position is the dominant TSS in stimulated cells. The role of alternative splicing in the 5′UTR has not been well studied. We found a similar 5′UTR gene organization in the human CysLTR1 gene, and similar gene structures were identified in other GPCR genes (20, 22, 23), suggesting that this kind of gene organization is not unique and may have functional significance. A GenBank database search revealed EST sequences matching our transcript VII (BX481095.1 derived from muscle, DA916197.1 from small intestine) and transcript V (DA860885.1 from placenta). Another transcript, representing our transcript IV, was identified in cDNA from brain, and some promoter activity has been reported in the region directly 5′ from exon 3 of CysLTR2 (24). However, we did not observe a single mRNA beginning in this region. All CysLTR2 transcripts identified by us originated in exon 1, in two closely localized regions between nt −53 and −289, where multiple TSS were detected. Most of the transcripts started in the proximal region, suggesting that this is the primary site of CysLTR2 transcription initiation. Gene reporter analysis performed in endothelial cells provided data pointing to a region between nt −168 and −442 as a putative basal promoter, with the highest activity present in the p224 construct. Similar to other GPCR promoters, CysLTR2 is TATA-less and contains several binding sites for transcription factors, such as AP1, SP1, and GATA. The murine CysLTR2 gene structure has been reported, (25), showing a similar gene organization, consisting of six exons, with five of them localized to 5′UTR. Interestingly, although we found a significant homology (>70%) between mouse and human promoter regions, no homology was detected between other exons localized to the 5′UTR. The murine CysLTR2 gene is also alternatively spliced, but only two different transcripts have been reported. Using the TRANSFAC database, several putative IFN-γ-induced transcription factor-binding motifs were detected in the CysLTR2 promoter, suggesting that the gene may be up-regulated by IFN-γ at the transcriptional level. To our surprise, there was no significant difference in reporter gene activity from all deletion CysLTR2 promoter constructs in IFN-γ-stimulated and control cells. We did not observe a change in CysLTR2 mRNA 1/2 after
IFN-γ stimulation either. As most of the data point to transcription as mechanism responsible for CysLTR2 up-regulation, it is possible that important IFN-γ response motifs or enhancers are localized further 5′ from the end of our longest promoter construct or within the introns and 5′UTR (52.8 kb). For example, such an intronic enhancer was shown to regulate Cox-1 expression in response to PMA (26). In agreement with our observation, it has been shown recently that IFN-γ can up-regulate CysLTR2 expression in cosinophils, but the mechanism has not been studied (27). It may be hypothesized that the similar mechanism of IFN-γ-mediated enhancement of cysLT signaling may apply to other cells expressing this receptor.

Little is known about cysLT signaling through CysLTR2, and functions subserved by this receptor have not been characterized yet. Our study showed that human endothelial cells express only CysLTR2, but not CysLTR1, and IFN-γ further up-regulates selectively CysLTR2. Thus, endothelial cells become a good model for studying CysLTR2 signaling and its role in vascular biology. A very recent microarray study of Uzonyi et al. (28) for the first time expressed this receptor.

mediated enhancement of cysLT signaling may apply to other cells expressing this receptor.


Disclosures

The authors have no financial conflict of interest.

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