Dominant Expression of the CysLT\textsubscript{2} Receptor Accounts for Calcium Signaling by Cysteinyl Leukotrienes in Human Umbilical Vein Endothelial Cells

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Objective—The objective of the present study was to identify and characterize the cell-surface receptors on human umbilical vein endothelial cells (HUVECs) that transduce calcium transients elicited by cysteinyl leukotrienes (CysLTs), potent spasmodic and proinflammatory agents with profound effects on the cardiovascular system.

Methods and Results—Using quantitative reverse transcription–polymerase chain reaction, we found that HUVECs abundantly express CysLT\textsubscript{2} R mRNA in vast excess (>4000-fold) of CysLT\textsubscript{1} R mRNA. Lipopolysaccharide, tumor necrosis factor-\textalpha, or interleukin-1\textbeta caused a rapid (within 30 minutes) and partially reversible suppression of CysLT\textsubscript{2} R mRNA levels. Challenge of HUVECs with BAY u9773, a specific CysLT\textsubscript{2} R agonist, triggered diagnostic Ca\textsuperscript{2+} transients. LTC\textsubscript{4} and LTD\textsubscript{4} are equipotent agonists, and their actions can be blocked by the dual-receptor antagonist BAY u9773, but not by the CysLT\textsubscript{1} R-selective antagonist MK571.

Conclusions—HUVECs almost exclusively express the CysLT\textsubscript{2} R. Furthermore, Ca\textsuperscript{2+} fluxes elicited by CysLT in these cells emanate from perturbation of the CysLT\textsubscript{2} R, rather than the expected CysLT\textsubscript{1} R. Hence, signaling events involving CysLT\textsubscript{2} R might trigger functional responses involved in the critical components of LT-dependant vascular reactions, which in turn have implications for ischemic heart disease and myocardial infarction. (Arterioscler Thromb Vasc Biol. 2003;23:e37-e41.)

Key Words: leukotrienes ■ endothelial cells ■ receptors ■ inflammation ■ artherosclerosis

The cysteinyl leukotrienes (CysLTs) are a family of powerful lipid mediators, typically formed by eosinophils, basophils, monocytes, and mast cells, which are involved in critical steps of inflammatory and allergic diseases, particularly in the respiratory tract and cardiovascular system.\textsuperscript{1} The CysLTs are biosynthesized from arachidonic acid and its oxygenation product, LTA\textsubscript{4} [5(\textit{S})-\textit{trans}-5,6-oxido-7,9-\textit{trans}-11,14-\textit{cis}-eicosatetraenoic acid], generated by the enzyme 5-lipoxygenase. LTA\textsubscript{4} is further conjugated with reduced glutathione by LTC\textsubscript{4} synthase to form LTC\textsubscript{4} [5(\textit{S})-\textit{hydroxy}-6(\textit{R})-S-glutathionyl-7,9-\textit{trans}-11,14-\textit{cis}-eicosatetraenoic acid], ie, the parent compound of the CysLTs (LTC\textsubscript{\textit{\alpha}}, LTD\textsubscript{\textit{\alpha}}, and LTE\textsubscript{\textit{\alpha}}). The biologic effects of CysLTs are signaled through 2 principal G protein–coupled receptors, designated CysLT\textsubscript{1} R and CysLT\textsubscript{2} R.\textsuperscript{2,3} The biologic role of the CysLT\textsubscript{2} R is presently not known, but its mRNA expression profile suggests that it might have important functions in the cardiovascular system.\textsuperscript{3–6}

Endothelial cells are strategically located at the interphase between the blood and parenchymal cells, where they take active part in physiologic and pathologic processes involving the vessel wall. Under inflammatory conditions, these cells are directly exposed to LTs that are formed by activated, adhering leukocytes or via transcellular routes involving platelets or endothelial cells themselves.\textsuperscript{7–9} The CysLTs in turn trigger a number of specific functional responses in endothelial cells, eg, synthesis of platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine), secretion of von Willebrand factor, and expression of P-selectin, all of which might contribute to vascular inflammation.\textsuperscript{10,11} Here we report that human umbilical vein endothelial cells...
(HUVECs) abundantly express the CysLT_{2R} in vast excess over the CysLT_{1R}. Moreover, CysLT_{2R} transduces Ca^{2+} signals elicited by CysLTs in these cells, which in turn might be linked to pathologic processes of the vessel wall.

**Methods**

**Materials**

LTC_{4} and LTD_{4} were from BioMol, whereas MK571 and BAY u9773 were kind gifts from Dr. Sven-Erik Dahlén, Karolinska Institutet, Stockholm, Sweden. Oligonucleotides were from Cybergene AB. Lipopolysaccharide (LPS) from Escherichia coli serotype O55:B5, tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and fura 2-AM were from Sigma Chemical Co.

**Cell Culturing**

HUVECs were obtained from vessels by collagenase treatment and were then cultivated and identified, as described. The cell viability was >95%, as judged by cell morphology, trypan blue exclusion, and analysis of lactate dehydrogenase release. Glass dishes were seeded with 10^5 HUVECs per well and grown to confluence.

**Analysis of mRNA by RT-PCR and Real-Time RT-PCR**

Preparation of total RNA and reverse transcription–polymerase chain reaction (RT-PCR) were performed essentially as described. In nested PCR analyses, 12 + 20 cycles (CysLT_{2R} mRNA) or 40 + 40 cycles (CysLT_{1R} mRNA) were used for the first set (5'-ATGGAGAGAAAATTTATGTC; 5'-AATAGAGAGGAGATTGGAAC) and second set (5'-ACCTTACGAAATAACAGC; 5'-CTTTACTGATCTGCTTTGC) of primers, respectively (Figure 1). One of 2 housekeeping genes (glyceraldehyde 3-phosphate dehydrogenase and β-actin) was amplified as an internal standard and tested for potential responsiveness to the cell stimulus before calculations. For semiquantitative analysis of PCR products, a fluorescent dye (Picogreen, Molecular Probes) was used according to the manufacturer’s instructions. The fluorescence was measured (excitation wavelength=485 nm; emission wavelength=538 nm) in a fluorometer (SpectraMax GeminiXS). For comparative RT-PCR, total RNA was diluted until the PCR signal for CysLT_{1R} was comparable to that of CysLT_{2R} in undiluted RNA, with the use of 40 + 40 cycles. To correct for differences in template efficiency, equal amounts of CysLT_{1R} and CysLT_{2R} DNA standards were subjected to PCR amplification, and the relative signal intensity was used for normalization. Real-time RT-PCR was performed on a commercially available amplification system (Rotor Gene 2000, Corbett Research Mortlake) with SYBR green (Roche Diagnostics) as the detection system and second set (5'-CTTTACTGATCTGCTTTGC) of primers, respectively (Figure 1). One of 2 housekeeping genes (glyceraldehyde 3-phosphate dehydrogenase and β-actin) was amplified as an internal standard and tested for potential responsiveness to the cell stimulus before calculations. For semiquantitative analysis of PCR products, a fluorescent dye (Picogreen, Molecular Probes) was used according to the manufacturer’s instructions. The fluorescence was measured (excitation wavelength=485 nm; emission wavelength=538 nm) in a fluorometer (SpectraMax GeminiXS). For comparative RT-PCR, total RNA was diluted until the PCR signal for CysLT_{1R} was comparable to that of CysLT_{2R} in undiluted RNA, with the use of 40 + 40 cycles. To correct for differences in template efficiency, equal amounts of CysLT_{1R} and CysLT_{2R} DNA standards were subjected to PCR amplification, and the relative signal intensity was used for normalization. Real-time RT-PCR was performed on a commercially available amplification system (Rotor Gene 2000, Corbett Research Mortlake) with SYBR green (Roche Diagnostics) as the detection system.

**Ca^{2+} Mobilization Experiments**

Mobilization of cytosolic calcium, Ca^{2+}, was monitored spectrophotometrically with the use of fura 2-AM, essentially as described. The results are given as the ratio of fluorescence between 340 and 380 nm, calibrated and calculated with commercially available software (Miracal, Life Science Resources Ltd), according to the recommendations of the manufacturer.

**Results and Discussion**

**Dominant Expression of CysLT_{2R} mRNA in HUVECs**

Nested RT-PCR analyses (12 + 20 cycles) of total RNA from HUVECs generated a robust signal, corresponding to CysLT_{2R} mRNA (793 bp), whereas no signal for CysLT_{1R} could be detected (Figure 1). However, when the number of cycles was increased to 40 + 40, a weak signal for CysLT_{2R} mRNA (947 bp) was obtained, as previously reported. To quantify the difference in mRNA levels, we made serial dilutions of the total RNA until the signal for CysLT_{2R} was comparable to that of CysLT_{1R} in undiluted RNA and then corrected the signal intensities for differences in template efficiency (Figure 1). With this technique, the ratio of CysLT_{2R} mRNA to CysLT_{1R} mRNA was calculated as >4000:1. In addition, real-time RT-PCR detected 2130 molecules of CysLT_{1R} mRNA and 4 molecules of CysLT_{2R} mRNA, indicating a 500-fold difference in mRNA levels (Figure 1). However, cautious interpretation is warranted, because the value for CysLT_{2R} transcripts was obtained by extrapolation outside the linear portion of the standard curve.
Rapid Suppression of CysLT2 R mRNA Levels by LPS, IL-1β, and TNF-α

Treatment of HUVECs with LPS (100 ng/mL), IL-1β (5 U/mL), or TNF-α (10 ng/mL) for 30, 60, and 120 minutes led to rapid (30 to 60 minutes) suppression (30% to 60%) of the CysLT2 R mRNA level (Figure 2). The effects of LPS were reversible, whereas those of IL-1β and TNF-α persisted after 120 minutes, with mRNA levels 20% and 40%, respectively, below those of controls, suggesting that these cytokines reduce the expression of CysLT2 R. The levels of CysLT1 R mRNA remained very low, and no significant alterations could be detected (data not shown).

Challenge of HUVECs With LTC4, LTD4, and BAY u9773 Elicits Ca2+ Transients That Are Diagnostic for CysLT2 R

In the absence of reliable antibodies against CysLT2 R, we used the natural agonists LTC4 and LTD4, together with the specific CysLT2 R agonist BAY u9773, to identify and functionally characterize CysLT2 R on HUVECs. Thus, stimulation of HUVECs with either LTC4 or LTD4 (100 nmol/L) elicited a Ca2+ response that peaked after ~5 to 10 seconds and lasted for nearly 1 minute (Figure 3). The 2 ligands were almost equipotent, in agreement with the typical ligand specificity of the CysLT2 R (LTC4 > LTD4). The levels of CysLT2 R mRNA remained very low, and no significant alterations could be detected (data not shown).
G protein–coupled surface receptors on HUVECs. Furthermore, stimulation of HUVECs with BAY u9773 elicited a significant Ca\(^{2+}\) response of almost the same strength as that of LTC\(_4\) and LTD\(_4\), thus providing direct evidence for the presence of functionally intact CysLT\(_R\) (Figure 3). Moreover, an initial challenge with LTD\(_4\) blocked the Ca\(^{2+}\) response to subsequent (1-minute) stimulation with BAY u9773 (Figure 3), indicating CysLT\(_R\) occupancy or desensitization.

### CysLT\(_R\) Is the Major Functional Receptor for CysLTs in HUVECs

At present, there is no selective CysLT\(_R\) antagonist available. However, BAY u9773 is not only a CysLT\(_R\) agonist (see Methods) but also a dual CysLT\(_R\) and CysLT\(_R\) antagonist. Therefore, we used BAY u9773 together with MK571, a selective CysLT\(_1\) R antagonist, to assess the role of CysLT\(_R\) in CysLT-induced Ca\(^{2+}\) signaling. Preincubation of HUVECs for 15 minutes with 1 \(\mu\)mol/L BAY u9773 completely blocked the Ca\(^{2+}\) responses to a subsequent stimulation with 100 nmol/L of either LTC\(_4\) or LTD\(_4\) (Figure 3). In contrast, MK571 (1 \(\mu\)mol/L, 15 minutes) was a very poor antagonist and could only partially, if at all, prevent subsequent Ca\(^{2+}\) mobilization by 100 nmol/L LTC\(_4\) or LTD\(_4\) (Figure 3). This pharmacologic profile, together with the similar agonistic potency of LTC\(_4\) and LTD\(_4\), as well as the high level of mRNA (cf Figure 1), strongly indicates that HUVECs almost exclusively express the CysLT\(_R\) and that this receptor accounts for the Ca\(^{2+}\) signals induced by CysLTs in these cells. It should be noted that we and others have previously reported that HUVECs express the CysLT\(_R\), albeit at a low level, unless the cells are subjected to long-term treatment with IL-1\(\beta\). In light of the results of the present study, it seems likely that CysLT\(_R\) on HUVECs lacks functional integrity and will not contribute significantly to Ca\(^{2+}\) signaling triggered by CysLTs.

### Potential Role of CysLT\(_R\) in the Cardiovascular System

CysLTs have profound effects in the vascular system and constict coronary vessels with a negative inotropic effect and reduced cardiac output. Furthermore, early metabolic studies have suggested that CysLTs play a role in cardiac ischemia and only very recently, pharmacologic, genetic, and immunohistochemical data were presented that indicate that 5-lipoxygenase and LTs are involved in atherosclerosis and ischemic heart disease. Furthermore, CysLTs, formed via transcellular routes along a leukocyte–endothelial cell axis, might elicit coronary vasospasm and inflammatory changes in the vasculature. For certain contraction-relaxation responses of the vessel wall, CysLT\(_R\) has been implicated in signal transduction, particularly during inflammatory states in the microvasculature, and CysLT\(_R\) mRNA is expressed in smooth muscle cells. Most likely, signaling via CysLT\(_R\) also contributes significantly to the cardiovascular effects of CysLTs, because mRNA has been identified in the heart, including Purkinje cells, smooth muscle cells, and heart muscle. Interestingly, Hui et al\(^{11}\) recently reported that in the mouse heart, CysLT\(_R\) mRNA was also found in certain endothelial cells, in line with our data for HUVECs. Moreover, CysLTs have been found to elicit several specific functional responses in endothelial cells, eg, synthesis of platelet-activating factor, secretion of von Willebrand factor, and surface expression of P-selectin. Hence, although the cardiovascular actions of CysLTs are complex and involve both the CysLT\(_R\) and CysLT\(_R\), our data demonstrate that CysLT\(_R\) is the dominating, functional receptor for CysLTs on HUVECs and might thus be involved in the propagation of local as well as systemic effects of these lipid mediators. Certainly, the CysLT\(_R\) appears to be an interesting target for pharmacologic intervention in cardiovascular diseases, in particular, ischemic heart disease and myocardial infarction.

### Acknowledgments

This work was financially supported by the Swedish Research Council (O3X-10350, 19X-05991), the Vårdal Foundation, the European Union (QLG1-CT-2001-01521), Konung Gustav V:s 80-årsfond, the Foundation for Strategic Research, the AFA Health Foundation, the Swedish Association against Rheumatism, the Swedish Heart and Lung Foundation, Systembolagetis Research Fund, and the funds of Uggla and Wiberg. The authors thank Andreas Habenicht for technical help and discussions.

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Arterioscler Thromb Vasc Biol. 2003;23:e37-e41; originally published online June 19, 2003;
doi: 10.1161/01.ATV.0000082689.46538.DF
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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