Differential Leukotriene Receptor Expression and Calcium Responses in Endothelial Cells and Macrophages Indicate 5-Lipoxygenase–Dependent Circuits of Inflammation and Atherogenesis

Katharina Lötzer,* Rainer Spanbroek,* Markus Hildner, Anja Urbach, Regine Heller, Ellen Bretschneider, Helen Galczenski, Jilly F. Evans, Andreas J.R. Habenicht

Objective—Inflammatory infiltrates and atherosclerotic lesions emerge when monocytes adhere to endothelial cells (ECs), migrate into the subendothelial space, and become macrophages (MΦs). Leukotrienes (LTs), products of 5-lipoxygenase, are powerful inflammatory mediators. 5-lipoxygenase MΦs have been shown to increase during atherogenesis, and LT receptor (LT-R) transcripts were identified in diseased arteries. To investigate LT-Rs in cells involved in inflammation and atherogenesis, we used the in vitro models of human umbilical vein ECs (HUVECs) and monocyte-derived MΦs.

Methods and Results—HUVECs primarily expressed transcripts of the cysteinyl (cys) LT 2-R, which was strongly upregulated by interleukin-4. By contrast, MΦs predominantly expressed transcripts of the cysLT 1-R. Calcium responses toward LTs revealed differential cysLT-R utilization by both cell types: HUVECs responded to both cysLTs, whereas MΦs preferentially responded to LTD4; HUVECs, but not MΦs, were resistant toward a cysLT 1-R antagonist, montelukast; cysLTs generated regular calcium oscillations in HUVECs that lasted >60 minutes, resulting in >500 oscillations per cell. By contrast, calcium elevations in MΦs returned to baseline within seconds and were nonoscillatory.

Conclusions—Our data raise the possibility that MΦ-derived LTs differentially activate cysLT 2-Rs via paracrine stimulation and cysLT 1-Rs via autocrine and paracrine stimulation during inflammation and atherogenesis. (Arterioscler Thromb Vasc Biol. 2003;23:e32-e36.)

Key Words: leukotriene receptors ■ endothelial cells ■ macrophages ■ inflammation ■ atherogenesis

L eukotriene B4 (LTB4) and the cysteinyl (cys) LTs, LTC4, LTD4, and LTE4, are potent, proinflammatory mediators.1,2 Four LT-activated, putative, 7 transmembrane G protein–coupled receptors have been characterized: 2 receptors for LTB4, namely BLT-Rs,3,4 and 2 receptors for LTD4 and LTC4, namely, cysLT-Rs.5,6 Whereas LTB4 mediates chemotaxis,3 cysLTs trigger a variety of tissue responses, including increases in vascular permeability.7 CysLT 1-R is primarily present in the spleen, blood leukocytes, and lung macrophages (MΦs),8 whereas cysLT 2-R is expressed in the heart and brain.8 However, the precise cell lineage–specific expression of LT-Rs, their mode of regulation, and the signaling pathways that they trigger remain to be determined. We observed transcripts of all 4 LT-Rs in human atherosclerotic lesions.8 These data raised the possibility that the 5-lipoxygenase (5-LO) pathway forms foci of inflammation within the arterial wall. Because human umbilical vein endothelial cells (HUVECs) and MΦs have been used to study inflammation and recent studies have associated the 5-LO pathway with experimental atherosclerosis,9–15 we examined their LT-R expression patterns and characterized their calcium responses.

Methods
Montelukast (Singulair) was a kind gift of Dr R.N. Young, Merck Frosst (Quebec, Canada). BAY u9773 was obtained from Biomol;
fura 2-AM, from Calbiochem; fluo 4-AM, from Molecular Probes; transforming growth factor-β (TGF-β), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and IL-4, from R&D Systems; LTs, from Cayman Chemicals; CD14 MicroBeads, from Miltenyi Biotech; and other reagents, from Sigma. Monocytes were purified from peripheral blood mononuclear cells by adherence or CD14 microbead adsorption and maintained at 1×10⁵ cells/mL for 5 to 9 days in RPMI-1640 medium plus 20% autologous human serum. HUVECs were cultured as described and used at passages 1 to 2. Carotid artery disease lesions were obtained after informed consent of the patients, as described. HUVECs or MΦs were loaded with fura 2-AM at 4 μmol/L, and changes in calcium ions were expressed as excitation ratios of 340 nm/380 nm at 510 nm. For single-cell measurements, cells were loaded with 2 μmol/L fluo 4-AM. Responses were recorded on a microscope (Axiovert 200M) equipped with a confocal laser scanning head (LSM510). Quantitative (real-time) reverse transcription–polymerase chain reaction (qRT-PCR) was performed as previously reported.

Results
Expression Patterns of LT-R Transcripts in HUVECs and MΦs
Determination of sequence-confirmed RT-PCR products of LT-Rs in HUVECs and MΦs indicated differential expression of transcript levels. Preliminary, nonquantitative RT-PCR analyses indicated that HUVECs showed significant cysLT₁-R and detectable BLT₁-R and BLT₂-R but no or low cysLT₂-R transcripts. By contrast, MΦs preferentially expressed cysLT₁-R transcripts and, to a lesser extent, cysLT₂-R, BLT₁-R, and BLT₂-R transcripts (Figure 1a). qRT-PCR demonstrated the magnitude of differential LT-R transcript expression in both cell types (when expressed as absolute transcripts/10⁵ glyceraldehyde 3-phosphate dehydrogenase [GAPDH] transcripts): HUVECs expressed cysLT₁-Rs in large excess when compared with cysLT₂-Rs (cysLT₁-Rs, 983±527; cysLT₂-R, 3±3; n=6). By contrast, MΦs expressed cysLT₁-Rs in large excess when compared with cysLT₂-Rs (cysLT₁-Rs, 6754±4507; cysLT₂-R, 156±123; n=8). Interestingly, carotid artery disease lesions that represent advanced atherosclerotic lesions (type V) according to the American Heart Association nomenclature expressed cysLT₁-Rs that were only 3-fold higher than cysLT₂-Rs (cysLT₁-R, 439±158; cysLT₂-R, 129±53; n=5). The numbers represent mean±SD and are highly significant: P<0.01 for cysLT₁-R versus cysLT₂-R in HUVECs and MΦs. These data show that HUVECs expressed cysLT₁-R transcripts at 2 orders of magnitude higher levels when compared with cysLT₂-R transcripts.

Cytokines implicated in inflammation and atherogenesis were examined for regulation of cysLT₁-R transcripts. IL-4, but not IL-1β, TGF-β1, or TNF-α (Figure 1b), caused upregulation of cysLT₁-R transcripts in a concentration- and time-dependent way (Figure 1c and 1d). Confluent HUVECs expressed ≈2-fold higher levels of cysLT₁-R transcripts when compared with their rapidly proliferating counterparts (Figure 1d, open columns). Because both IL-4 and cell density contributed to cysLT₁-R transcript upregulation in an additive way, this amounted to a 7-fold increase at 48 and 72 hours. Moreover, levels of cysLT₁-R transcript decreased with increasing passage numbers.

Figure 1. Differential LT-R expression in HUVECs and MΦs. HUVECs and MΦs were examined for LT-R transcript expression by RT-PCR (a) or qRT-PCR (b–d) as described in Methods. HUVECs were seeded at 1 to 2×10⁵ cells/cm² and maintained for 3 days until they reached subconfluence. Cells were stimulated with IL-4 at 50 ng/mL; TGF-β1 at 5 ng/mL; IL-1β at 100 U/mL, and TNF-α at 100 U/mL for 24 hours (b); increasing concentrations of IL-4 for 48 hours (c); or 10 ng/mL IL-4 for increasing periods of time (d). Control cells (open columns) received carrier for 24 hours (b) or 48 hours (c). IL-4-stimulated cells were different from controls at P<0.05 (b), P<0.02 (at 1 nmol/L IL-4; c), P<0.001 (at 10 nmol/L IL-4; d) or P<0.02 (48 hours; d) (all by Student’s t test). TNF-α treatment significantly reduced transcript numbers of cysLT₁-R (P<0.04; n=3). Experiments were performed at least twice, and values were normalized to nanograms of RNA; there was no change of GAPDH transcripts per nanogram RNA on cytokine treatment.
Calcium Responses Indicate Differential cysLT-R Utilization by HUVECs and MΦs

In receptor-transfected cells, cysLT₁-R has been shown to bind LTC₄ with lower affinity than LTD₄, whereas the cysLT₂-R binds LTC₄ and LTD₄ with similar affinities, but functional studies of cysLT₁-R versus cysLT₂-R in HUVECs have not yet been reported.⁵,⁶ Initially, calcium responses toward LTC₄/LTD₄ of HUVECs, whereas the selective cysLT₁-R antagonist montelukast (Singulair) failed to inhibit calcium transients (Figure 2a and 2b). Moreover, BAY u9773, a partial cysLT₂-R agonist,¹⁷ induced a moderate response (Figure 2b). By contrast, 50 nmol/L LTD₄ triggered robust calcium responses in MΦs, whereas LTC₄ at 50 nmol/L was inactive, and LTD₄-dependent calcium responses were completely blocked by montelukast (Singulair; Figure 2c). These data are consistent with and extend studies reported by others that cysLTs triggers calcium elevations in cultured HUVECs and MΦs.¹⁸⁻²⁰ Thus, our data demonstrate for the first time that the dominant, functional cysLT-R in HUVECs is the cysLT₂-R. They establish HUVECs as a culture system in which selective activation of the cysLT₂-R for the biology of ECs can be studied.

Calcium Elevations in HUVECs and MΦs Reveal Differences in Kinetics, Amplitude, Duration, and Oscillatory Patterns

We noted that the initial rise of calcium ions in HUVECs was followed by a pronounced, sustained component, whereas that in MΦs rapidly returned to near-baseline levels within seconds (compare Figure 2a, 2b, and 2c). We next determined calcium responses in single cells. In HUVECs, the rapid initial LTC₄/D₄-dependent rise in calcium ions was followed by oscillations that, once established, proceeded at regular intervals (Figure 2d). These data confirmed studies by Datta et al.,¹⁸ who demonstrated that cysLTs trigger oscillatory calcium rises in HUVECs. By contrast, in MΦs, LTD₄ induced an initial increase in calcium ions, which rapidly returned to baseline levels (Figure 2e) and was only rarely followed by single or irregular oscillations. These data show that oscillations are not invariably associated with the action of LTs on target cells, although they appear to depend on both the LT-R subtype and/or the target cell. We next initiated more detailed studies of the pharmacology, LT-R dependence, regularity, duration, intracellular location, variability, and dependence on culture conditions of oscillations in HUVECs. The frequency of oscillations did not vary considerably in individual HUVECs for extended periods of time,
although variability was noted between individual HUVECs. LT-dependent oscillations in HUVECs originated in subcellular areas in the cytoplasm (Figure 2f, arrow). Increases in calcium occurred in both the cytosol and the nucleus, whereby the nucleus was affected secondary to cytoplasmic calcium waves (Figure 2f). Once generated in one EC, cysLT-triggered calcium waves appeared to be transferred to other ECs, thus generating global intercellular calcium waves, probably through gap junctions. This interpretation was supported by inspection of slow-motion recordings of oscillations across intact portions of the monolayer that revealed areas of ECs in which coordinated calcium oscillations originated in calcium “puffs” of a single EC were observed. Importantly, calcium oscillations were a response to cysLT2-R activation, because montelukast failed to eliminate them (not shown). Moreover, calcium oscillations were dependent on extracellular calcium, because EGTA did not prevent the initial rise in calcium ions but led to an immediate cessation of ongoing oscillations. Culture parameters also influenced characteristics of calcium oscillations: a higher percentage of early-passage HUVECs responded to low concentrations of LTC4, the oscillation periods of HUVECs persisted for >60 minutes, resulting in >500 oscillations/h per HUVEC, whereas the response to another G protein–coupled receptor agonist, thrombin, was short-lived and subsided within seconds.

**Discussion**

HUVECs principally expressed cysLT2-Rs, which were up-regulated 7-fold by a combination of IL-4 and cell density (Figure 1). By contrast, HUVECs did not express significant numbers of cysLT1-Rs (Figures 1a, 2a, and 2b), yet MΦs principally expressed cysLT1-Rs (Figures 1a and 2c). Because selective cysLT-R antagonists are not yet available, these data establish HUVECs as the first bona fide cell system in which the signaling pathway of the cysLT2-R can be studied without interfering with cysLT1-R–dependent signaling. The cysLT-R expression patterns in HUVECs and MΦs yielded calcium transients with very different properties: HUVECs and MΦs differed in their sensitivities toward LTC4 and LTD4 (Figure 2); response to LT-R antagonists; kinetics, regularity, and duration of calcium increases; and oscillatory versus nonoscillatory patterns.

When previous and the present data on expression of the 5-LO pathway and LT-Rs in ECs, MΦs, and advanced human atherosclerotic lesions are considered, our data raise the possibility that leukocyte/EC/T-cell interactions might constitute inflammatory “circuits” through at least 2 major mechanisms: MΦ-derived LTs might activate cysLT2-Rs via autocrine mechanisms and EC cysLT2-Rs and T-lymphocyte cysLT1-Rs, via paracrine mechanisms during physiologic leukocyte trafficking, inflammation, and atherogenesis (Figure 3).

**Acknowledgments**

This study was supported by grants of the Deutsche Forschungsgemeinschaft (Ha 1083/1–3/1–2), the European Union research network (QLG1-CT-2001-01521), and the Interdisziplinäres Zentrum für Klinische Forschung, Jena. We thank C. Ströh, M. Franke, and E. Teuscher for expert technical assistance.

**References**


Differential Leukotriene Receptor Expression and Calcium Responses in Endothelial Cells and Macrophages Indicate 5-Lipoxygenase–Dependent Circuits of Inflammation and Atherogenesis

Katharina Lötzer, Rainer Spanbroek, Markus Hildner, Anja Urbach, Regine Heller, Ellen Bretschneider, Helen Galczenski, Jilly F. Evans and Andreas J.R. Habenicht

Arterioscler Thromb Vasc Biol. 2003;23:e32-e36; originally published online June 19, 2003; doi: 10.1161/01.ATV.0000082690.23131.CB

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 2003 American Heart Association, Inc. All rights reserved.

Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://atvb.ahajournals.org/content/23/8/e32

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/