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Cysteinyl leukotriene signaling through perinuclear CysLT1 receptors on vascular smooth muscle cells transduces nuclear calcium signaling and alterations of gene expression

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Abstract  Leukotrienes are pro-inflammatory mediators that are locally produced in coronary atherosclerotic plaques. The response induced by cysteinyl leukotrienes (CysLT) in human coronary arteries may be altered under pathological conditions, such as atherosclerosis. The aim of the present study was to elucidate cysteinyl leukotriene signaling in vascular smooth muscle cells (SMCs) and the effects of inflammation on this process. Immunohistochemical analysis of human carotid endarterectomy samples revealed that the CysLT1 leukotriene receptor was expressed in areas that also stained positive for α-smooth muscle actin. In human coronary artery smooth muscle cells, lipopolysaccharide significantly upregulated the CysLT1 receptor and significantly enhanced the changes in intracellular calcium induced by leukotriene C4 (LTC4). In these cells, the CysLT1 receptor exhibited a perinuclear expression, and LTC4 stimulation predominantly enhanced nuclear calcium increase, which was significantly inhibited by the CysLT1 receptor antagonist MK-571. Microarray analysis revealed, among a number of significantly upregulated genes after 24 h stimulation of human coronary artery smooth muscle cells with LTC4, a 5-fold increase in mRNA levels for plasminogen activator inhibitor (PAI)-2. The LTC4-induced increase in PAI-2 expression was confirmed by real-time quantitative PCR and ELISA and was inhibited by the CysLT1 receptor antagonist MK-571 and by calcium chelators. In summary, pro-inflammatory stimulation of vascular SMCs upregulated a perinuclear CysLT1 receptor expression coupled to nuclear calcium signaling and changes in gene expression, such as upregulation of PAI-2. Taken together, these findings suggest a role of nuclear CysLT1 receptor signaling in vascular SMCs inducing gene expression patterns associated with atherosclerosis.

Keywords Atherosclerosis · Eicosanoids · Inflammation · Lipoxigenase · PAI-2

Introduction

Although initially identified as targets in the treatment of asthma, recent findings have brought attention to leukotrienes (LTs) as potential mediators of cardiovascular disease [1], such as atherosclerosis [2], abdominal aortic aneurysms [3], and aortic stenosis [4]. Human coronary artery atherosclerotic lesions are a source of cysteinyl LTs (i.e., LTC4, D4, and E4) [5], and urinary levels of LTE4 are increased in acute coronary syndromes [6], implicating these mediators in coronary atherosclerosis and plaque instability. The notion of cysteinyl LTs as potential effectors of atherosclerosis has also received support from animal models showing beneficial effects on atherosclerosis burden [7, 8] and...
in the nature of the increased CysLT we have investigated this idea, with the aim to quantify and in males [9]. In human carotid atherosclerotic lesions, a 3-fold higher CysLT receptor expression compared with the CysLT receptor has been reported [10], although the cellular localization of these receptors has not yet been resolved. The signaling through the CysLT receptor subtypes have been widely studied in the context of bronchoconstriction and asthma [10]. Recently, the CysLT receptor antagonist montelukast was shown to be associated with a decreased risk of ischemic stroke and a decreased risk of myocardial infarction in males [12]. Although the latter report provides a first indication of beneficial effects of clinically used anti-leukotriene drugs, the exact role of CysLT receptor signaling in atherosclerosis remains to be established.

In addition to being bronchoconstrictors, cysteinylic LTs are also potent vasoconstrictors in the human lung [13]. Their role in the coronary vasculature, however, can be said to be contextually antithetical, as healthy human coronary arteries are unresponsive to cysteinylic LTs, but a contractile response to either LTC4 or LTD4 is observed in atherosclerotic coronary arteries [14, 15]. Although no previous study has addressed the mechanism for this differential sensitivity between healthy and atherosclerotic vessels, it is interesting to note that these leukotriene-induced contractions are inhibited by CysLT receptor antagonists [14]. The latter observation is, however, in contrast to the dominant CysLT2 receptor expression in human coronary artery smooth muscle cells (SMCs) [16].

With the notion in mind that a local production of cysteinylic LTs within the atherosclerotic lesion could potentially activate CysLT receptors within the vascular wall, we engaged in this study with the hypothesis that the inflammatory environment of atherosclerosis could lead to an upregulation of CysLT1 receptors on vascular SMCs. Here, we have investigated this idea, with the aim to quantify and describe the nature of the increased CysLT1 signaling. Further, we have explored the potential downstream effects of this phenomenon on both intracellular signaling and gene expression in vascular SMCs in an effort to link CysLT1 receptor signaling to atherogenic properties of SMCs in the context of inflammation and leukotriene stimulation.

**Methods**

**Cell culture**

Human coronary artery SMC purchased from Clonetics (Cambrex Bio Science, Walkersville, MD, USA) were cultured in SmGM2 kit medium as previously described [17] and harvested for experiments between passages 5 and 8. Cells were seeded in six-well plates (10^5 cells/well) containing SmGM2 48 h before the respective treatment, which was replaced with Dulbecco’s modified Eagle’s medium supplemented with 2 % fetal calf serum (starvation medium) 24 h before experiments. Subsequently, cells were incubated in the absence or presence of lipopolysaccharide (LPS; from Sigma, 10 μg/mL), interleukin-6 (IL-6; from Peprotech, 20 ng/mL), interferon-γ (IFN-γ; from Peprotech, 20 ng/mL), or tumor necrosis factor-α (TNF-α; from Peprotech, 10 ng/mL). Duration of treatment was experiment-dependent, as described in detail below. Cells were treated with various substances across experiments, including LTC4, LTD4, and LTE4 (from Cayman Chem, Ann Arbor, MI, USA; 1 μM), the CysLT1 receptor antagonist MK571 (from Cayman; 1 μM), ethylene glycol tetraacetic acid (EGTA; from Sigma, 5 mM), and 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxy-methyl ester) (BAPTA-AM; from Invitrogen, 10 μM).

**Immunostainings**

Atherosclerotic vascular tissue was collected from eight patients undergoing carotid endarterectomy. All experiments were performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and were approved by the local ethic committee (reference number 02/147). All persons gave their informed consent prior to their inclusion in the study. Immunofluorescent stainings were performed on acetone-fixed frozen sections of carotid endarterectomies and on human coronary artery SMCs cultured in LabTek slides after fixation and permeabilization with acetone–methanol. Rabbit anti-human CysLT1 receptor (from Cayman Chem) and mouse anti-human α-smooth muscle actin (from DAKO) were used as primary antibodies. Isotype-specific either DyLight 594 or DyLight 488-conjugated secondary antibodies (from Vector) were used, and the nuclei were counterstained with 4′,6-diamino-2-phenylindol (DAPI; Vector). Images were captured with confocal microscope Leica DMI.

**Calcium signaling experiments**

SMCs incubated for 48 h in the absence or presence of LPS (10 μg/mL) were washed and loaded with the fluorescent Ca2+ indicator fluo-3. The cells were subsequently stimulated with LTC4 (1 μM, 30 min at room temperature) in either Tyrode’s solution (composition, millimolars: NaCl 121, KCl 5.0, NaHCO3 24, CaCl2 0.5, MgCl2 0.4, NaH2PO4 0.4, EDTA0.1, and glucose 5.5) gassed with 5 % CO2 in O2 or Tyrode’s solution containing the CysLT1 receptor antagonist MK571 (1 μM). Changes in [Ca2+]i were recorded using a
BioRad MRC 1024 confocal microscopy unit attached to a Nikon Diaphot 200 inverted microscope with a Nikon Plan Apo ×20 or ×60 oil immersion objective (N.A.1.3), as previously described [18].

RNA extraction, cDNA synthesis, and TaqMan real-time PCR

Total RNA was extracted using RNeasy Mini kit (from Qiagen, Hilden, Germany) with an on-column DNase digestion step. RNA quantity was assessed using a Nanodrop ND-1000 microvolume spectrophotometer (Thermo Fisher Scientific), and RNA quality was assessed by a Bioanalyzer capillary electrophoresis system (Agilent Technologies, Palo Alto, CA, USA). First-strand cDNA was synthesized using Superscript II (Invitrogen, Carlsbad, CA, USA) with random hexamers according to the manufacturer’s instructions. Quantitative TaqMan PCR was performed on a 7900HT Fast Real-Time PCR system (Applied Biosystems) with primer/probe pairs that were obtained using Assay-on-Demand™ from Applied Biosystems for human CysLT1 (Hs00272624_s1) and Plasminogen Activator Inhibitor 2 (PAI-2/SERPIN B2; Hs01010736_m1). Levels of mRNA were normalized to expression levels of cyclophilin A (Hs99999904_m1), which previously has been determined as an appropriate housekeeping gene in these cells [17].

Microarray analysis

Microarray analysis experiments were performed on RNA derived from three separate SMC culture experiments, using either Agilent one-color whole human genome (44 K) kit (Agilent Technologies, Redwood City, CA, USA; n=2) or the Affymetrix Human Genome U133 Plus 2.0 array (n=1). Microarrays were analyzed with the Agilent high-resolution microarray scanner. Data were subsequently uploaded to GeneSpring GX10 (Agilent technologies) and analyzed using advanced analysis workflow for the Agilent one-color arrays. The set of data was normalized according to recommendations by GeneSpring for one-color arrays. (http://www.chem.agilent.com/cag/bsp/products/gsgx/manuals/GeneSpring-manual.pdf). Variability between chips was accounted for by applying a shift to the 75th percentile (dividing all measured signals by a 75th percentile value). Per-gene normalization was performed by bringing the baseline to the median of all samples. Probe sets were firstly filtered by confidence of detection, where genes that were not confidently detected in any sample were excluded from further analysis. Further filtering based on expression discarded any genes where less than 100 % of samples in either the relapse or diagnosis condition had expression values below the 20th percentile. The most differentially expressed genes, defined as those with an uncorrected P value of <0.05 and demonstrating a fold change in expression of 2.0 or greater, were selected for analysis. The list of 90 genes generated was subsequently compared to data from the Affymetrix arrays, and the genes of interest were verified in terms of direction of regulation. Genes meeting all these criteria are presented in Table 1. The 45 genes listed were submitted to Ingenuity® pathway analysis for prediction of canonical pathways and functional gene networks affected by the significant differential expression of these genes.

ELISA

PAI-2 ELISA was carried out on supernatants from untreated SMCs and SMCs treated LTC4 (1 μM) for 24 h using IMUBIND® PAI-2 ELISA kit (from American Diagnostica GmbH, Pfungstadt, Germany) according to manufacturer’s protocol.

Data analysis

All results are expressed as mean±SE. Statistically significant differences were determined by either a Student’s t test (for pair-wise comparisons) or a one-way analysis of variances, followed by Holm–Sidak post hoc test, for multiple comparisons, using Sigma Stat software. A P value of less than 0.05 was considered significant.

Results

CysLT1 receptor expression on vascular SMC

Immunohistochemical staining showed colocalization of the CysLT1 receptor protein with markers for SMC (α-smooth muscle actin) in human atherosclerotic lesions (Fig. 1). In human coronary artery SMCs, the transcriptional levels of the CysLT1 receptor were time-dependently increased by LPS, IL-6, and IFN-γ (Fig. 2). Fluorescent immunostainings revealed a predominantly perinuclear localization of the CysLT1 receptor in human coronary artery SMCs compared with α-smooth muscle actin, which stained positive in the whole cytoplasm (Fig. 3). The CysLT1 receptor in some cases demonstrated nuclear inclusions, as indicated by arrows in Fig. 3.

LTC4-induced nuclear calcium signaling in vascular SMCs

To evaluate whether CysLT1 receptors expressed on vascular SMC were functional, calcium changes in human coronary artery SMC were studied using the fluorescent Ca2+ indicator fluo-3 (Fig. 4a). LTC4 induced a dose-dependent increase in intracellular calcium, which was predominantly located in the nucleus (Fig. 4b). The LTC4-induced calcium increase was significantly higher in LPS-treated cells.
| Gene ID   | Probe ID | Gene name                                                                 | Mean fold change | Direction | P value   |
|----------|----------|---------------------------------------------------------------------------|------------------|-----------|-----------|
| CSF3     | A_23_P501754 | Colony stimulating factor 3                                               | 15.88            | Up        | 0.04128   |
| IL24     | A_23_P51951   | Interleukin 24                                                            | 15.11            | Up        | 0.04918   |
| SERPINB2 | A_23_P153185  | Serine proteinase inhibitor 2 (plasminogen activator inhibitor 2)          | 5.46             | Up        | 0.03882   |
| NEFM     | A_24_P264832  | Neurofilament medium polypeptide                                          | 4.89             | Up        | 0.01537   |
| GJA1     | A_23_P93591   | Gap junction protein. Alpha 1                                              | 4.74             | Up        | 0.00931   |
| IL1A     | A_23_P72096   | Interleukin 1 alpha                                                       | 4.15             | Up        | 0.01960   |
| CXCR4    | A_23_P102000  | Chemokine (C-X-C motif) receptor 4                                        | 3.94             | Up        | 0.00170   |
| TGFβ2    | A_24_P95070   | Tissue factor pathway inhibitor 2                                         | 3.90             | Up        | 0.02599   |
| CCND1    | A_24_P124550  | Cyclin D1                                                                 | 3.28             | Up        | 0.04980   |
| CXCL3    | A_24_P183150  | Chemokine (C-X-C motif) ligand 3                                           | 3.27             | Up        | 0.02149   |
| ABCG1    | A_23_P66297   | ATP-binding cassette. Sub-family G. Member 1                               | 3.03             | Up        | 0.00178   |
| HAS1     | A_23_P27400   | Hyaluronan synthase                                                       | 2.85             | Up        | 0.03572   |
| MMP3     | A_23_P161698  | Matrix metalloproteinase 3                                                | 2.70             | Up        | 0.01282   |
| PITPNC1  | A_24_P772103  | Phosphatidylinositol transfer protein. Cytoplasmic 1                      | 2.55             | Up        | 0.03758   |
| KITLG    | A_23_P204654  | KIT ligand                                                                | 2.49             | Up        | 0.00730   |
| FUBP3    | A_23_P435833  | Far upstream element (FUSE) binding protein 3                             | 2.46             | Up        | 0.00619   |
| NPTX1    | A_23_P124905  | Neuronal pentraxin 1                                                      | 2.45             | Up        | 0.00504   |
| PCSK1    | A_23_P213508  | Proprotein convertase subtilisin/kexin type 1                             | 2.41             | Up        | 0.04376   |
| ADSS     | A_23_P859     | Adenylosuccinate synthase                                                  | 2.40             | Up        | 0.01023   |
| TGFβ2    | A_23_P393620  | Tissue factor pathway inhibitor 2                                         | 2.38             | Up        | 0.01401   |
| NPR3     | A_23_P327451  | Natriuretic peptide receptor C/guanylate cyclase C                         | 2.33             | Up        | 0.01309   |
| SPRED1   | A_23_P54460   | Sprouty-related. Evh1 domain containing 1                                 | 2.30             | Up        | 0.02536   |
| PITPNC1  | A_23_P84189   | Phosphatidylinositol transfer protein. Cytoplasmic 1                      | 2.29             | Up        | 0.01626   |
| NKAIN1   | A_23_P51376   | Na+/K+ transporting ATPase interacting 1                                  | 2.26             | Up        | 0.02838   |
| SOCS4    | A_24_P90637   | Suppressor of cytokine signaling 4                                        | 2.23             | Up        | 0.00918   |
| TMTC3    | A_24_P944222  | Transmembrane and tetraacidopeptide repeat containing 3                  | 2.22             | Up        | 0.04956   |
| CACNG8   | A_32_P61693   | Calcium channel. Voltage-dependent. Gamma subunit 8                       | 2.19             | Up        | 0.00480   |
| RRN3     | A_23_P206877  | RNA polymerase I-specific transcription initiation factor RRN3            | 2.15             | Up        | 0.00613   |
| APOBEC3F | A_23_P369696  | Apolipoprotein B mRNA editing enzyme. Catalytic polypeptide-like 3F       | 2.12             | Up        | 0.03633   |
| NFE2L3   | A_24_P136653  | Nuclear factor (erythroid-derived 2)-like 3                              | 2.08             | Up        | 0.00912   |
| MYH11    | A_24_P70183   | Myosin heavy chain 11 (smooth muscle)                                     | 10.44            | Down      | 0.02218   |
| MYH11    | A_23_P206920  | Myosin heavy chain 11 (smooth muscle)                                     | 9.66             | Down      | 0.01751   |
| SCR1     | A_23_P167159  | Stimulator of chondrogenesis                                              | 4.73             | Down      | 0.01807   |
| TDP52L1  | A_23_P31143   | Tumor protein D52-like 1                                                 | 4.64             | Down      | 0.01830   |
| CARD9    | A_23_P500433  | Caspase recruitment domain family. Member 9                               | 3.54             | Down      | 0.00009   |
| CLIC3    | A_23_P254654  | Chloride intracellular channel 3                                          | 3.53             | Down      | 0.00765   |
| KRT7     | A_23_P381945  | Keratin 7                                                                 | 3.37             | Down      | 0.03569   |
| PCK2     | A_23_P128817  | Phosphoenolpyruvate carboxykinase 2 (mitochondrial)                      | 2.66             | Down      | 0.02696   |
| CTAGE1   | A_24_P124805  | Cutaneous T cell lymphoma-associated antigen 1                            | 2.57             | Down      | 0.01412   |
| GLI1     | A_23_P105251  | GLI family zinc finger 1                                                 | 2.56             | Down      | 0.01105   |
| SLCA17   | A_23_P325562  | Solute carrier family 1. Member 7                                        | 2.53             | Down      | 0.00758   |
| TGMI     | A_23_P65618   | Transglutaminase 1                                                        | 2.45             | Down      | 0.00203   |
| IL17RD   | A_32_P188860  | Interleukin 17 receptor D                                                 | 2.39             | Down      | 0.01570   |
| ZNF467   | A_23_P59470   | Zinc finger protein 467                                                  | 2.19             | Down      | 0.00169   |
| LIM2S    | A_23_P142796  | LIM and senescent cell antigen-like domains 2                            | 2.05             | Down      | 0.01940   |

Note that some genes may be listed twice due to significant differences detected by independent probes.
compared with untreated cells (Fig. 4c). In LPS-treated cells, the LTC₄-induced increase in nuclear calcium was significantly inhibited by the CysLT₁ receptor antagonist MK571 (Fig. 4c). The time course of the LTC₄-induced calcium increase in the nuclear and cytosolic compartments is shown in Fig. 4d. The increase in nuclear calcium preceded the increase in cytosolic calcium (Fig. 4d).

LTC₄-induced gene expression in vascular SMCs

The genes most significantly differentially expressed in response to LTC₄ (1 μM) in LPS-primed human coronary artery SMCs are presented in Table 1. PAI-2 (SERPIN B2), a member of the serine protease inhibitor superfamily, presented as one of the most significantly upregulated genes in the microarray analysis. This finding was confirmed by quantitative PCR (Fig. 5a), and in addition, increased PAI-2 protein levels were detected in the supernatant derived from LTC₄-stimulated human coronary artery SMCs, compared with unstimulated cells (Fig. 5b). The increased mRNA levels of PAI-2 induced by LTC₄ (1 μM) were mimicked by LTD₄ (1 μM) but not LTE₄ (1 μM) and significantly inhibited by the CysLT₁ receptor antagonist MK 571 (1 μM; Fig. 5a). In addition, the LTC₄-induced increase in PAI-2 mRNA was abolished by the removal of intra- and extracellular calcium, through experiments performed in the presence of BAPTA-AM and EGTA (Fig. 5c).

Ingenuity pathway analysis identified several functional gene networks predicted to be significantly affected by LTC₄ stimulation as based on the 45 genes determined to be differentially expressed through our microarray analysis (Table 1). The highest-scoring network (with a score of 35, equating to a fishers’ exact test score of 1×10⁻35) containing 15 genes from Table 1 is suggested to be implicated in cellular movement and hematopoietic system function and development. An outline of the network is shown in Supplementary Fig. 1.

Discussion

The results of the present study showed an upregulation of predominantly perinuclear CysLT₁ receptors in vascular SMCs under inflammatory conditions which was associated with increased nuclear calcium signaling and changes in gene expression. Taken together, these findings suggest a role of nuclear CysLT₁ receptor signaling in vascular...
SMCs inducing gene expression patterns associated with atherosclerosis.

Previous studies have suggested a dominant expression of the leukotriene CysLT<sub>2</sub> receptor subtype in human
coronary arteries [16]. In the present study, priming of vascular SMCs with LPS upregulated CysLT₁ receptor mRNA and enhanced LTC₄-induced effects. Similar findings have been reported in endothelial cells, which under resting conditions exhibit a dominant CysLT₂ receptor, but in which prolonged exposure to LPS or pro-inflammatory cytokines upregulate CysLT₁ receptor expression [19]. In the present study, CysLT₁ receptor expression was also upregulated in SMCs by IL-6 and by prolonged exposure to IFN-γ. Taken together, these observations suggest that a pro-inflammatory environment, such as atherosclerosis, may induce CysLT₁ receptor expression within the vascular wall. In support of the latter notion, it has been shown that LTC₄ induces contractions of atherosclerotic but not healthy coronary arteries [14, 15] and that CysLT₁ receptor signaling, but not CysLT₂ receptor signaling, is coupled to vasoconstriction in isolated systemic vessels [20]. LTC₄ has also been associated with SMC proliferation and the shift of SMCs into a synthetic phenotype [21], which is in line with findings that CysLT₁ receptor antagonism inhibits intimal hyperplasia after vascular injury in mice [9].

The present study is the first demonstration of a perinuclear localization of functional CysLT₁ receptors in vascular SMCs and a leukotriene-induced nuclear calcium signaling in these cells. These findings are nevertheless consistent with other G-protein-coupled receptors in vascular SMCs. For example, the ETₐ endothelin receptor and the AT₁ angiotensin II receptor exhibit a perinuclear localization in vascular SMCs coupled to nuclear calcium signaling [22–24]. In addition, we have recently demonstrated a perinuclear localization of the CysLT₁ receptor in valvular interstitial cells derived from human aortic valves [4], corroborating prior observations of CysLT₁ receptor expression at the outer nuclear membrane in intestinal epithelial cells [25]. In both of these cell types, leukotriene stimulation induces an increase in nuclear calcium [4, 25]. The present study extends those findings by demonstrating an enhanced nuclear calcium increase in response to LTC₄ after priming of cells with LPS and that the increase in nuclear calcium preceded the increase in cytosolic calcium. Whereas the present study cannot definitely conclude whether the subcellular CysLT₁ receptor localization represents an internalization process, previous studies support a translocation of CysLT₁ receptors between different cellular compartments, including nuclear inclusions [26].

Nuclear calcium is a key regulator of gene expression [27], and in line with this notion, LTC₄ induced significant changes in expression of several genes in the present study. Of particular interest was the appearance of PAI-2 as one of the most significantly upregulated genes in the microarray analysis. This was confirmed with qPCR analysis, and ELISA measures in addition showed that PAI-2 protein secretion from human coronary artery SMCs incubated 24 h in the absence (Contr) or presence of LTC₄ (1 μM). *P<0.05 vs. Contr (n=7). c Increase in PAI-2 mRNA levels induced by LTC₄ (1 μM) in human coronary artery SMC incubated in the absence (Contr) or presence of either BAPTA-AM or EGTA. *P<0.05 vs. Contr (n=3–6).
coronary artery SMCs was increased by LTC₄ stimulation. PAI-1 and PAI-2 are members of the serine proteinase inhibitor family and act as important inhibitors of fibrinolysis by interfering with the plasminogen system. PAI-1 is induced by atherogenic stimuli in vascular SMCs and may participate in cell growth and matrix degradation associated with atherosclerosis [28]. In addition, using cDNA representational difference analysis, PAI-2 has previously been identified as one of the most differentially expressed genes in atherosclerotic lesions compared with normal vessels, with elevated PAI-2 expression preferentially observed in unstable carotid plaques [29]. In addition, another study using serial analysis of gene expression in human vascular SMCs also identified PAI-2 as one of the most upregulated genes in response to conditioned media derived from macrophages activated by oxidized low-density lipoprotein [30]. Finally, immunohistochemical analysis of human atherosclerotic lesions has also confirmed that vascular SMCs stain positive for PAI-2 [29, 30]. In addition to acting as a plasminogen activator inhibitor, PAI-2 may serve as a regulator of Th1 immune responses through the modulation of cytokine-induced responses [31]. Furthermore, PAI-2 may be associated with the process of wound healing post-plaque rupture [29].

The LTC₄-induced increase in PAI-2 mRNA was abolished when experiments were performed in the presence of calcium chelators, suggesting a calcium dependent upregulation of PAI-2. The latter notion is supported by previous studies showing that angiotensin II is a potent inducer of PAI-2 in vascular SMCs through the AT₁ receptor [32], which is in line with a perinuclear AT₁ receptor localization and an EGTA-sensitive nuclear calcium signaling induced by angiotensin II [33]. Ingenuity pathway analysis revealed a significant number of LTC₄-upregulated genes to be implicated in a functional gene network linked to hematopoietic system function and cellular movement. Of note in this network was the involvement of cAMP response element binding protein (CREB), a transcription factor and member of the leucine zipper family of DNA binding proteins, which is known to be activated by nuclear calcium [27]. Furthermore, the PAI-2 gene promoter region contains a binding site for CREB (~1,319 bp), and this transcription factor has been shown to be associated with the induction of PAI-2 expression [34]. Taken together, these findings suggest that LTC₄-induced changes in gene expression may be induced through an increase in nuclear calcium leading to CREB activation. However, the pathway analysis also revealed other pathways that may be involved in LTC₄-induced gene expression, such as the NF-κB signaling pathway which has been previously shown to be activated after CysLT₁ receptor ligation in leukocytes [10].

In summary, we have shown that pro-inflammatory stimulation of vascular SMCs enhances perinuclear CysLT₁ receptor expression coupled to nuclear calcium signaling and results in changes in gene expression, such as upregulation of PAI-2. Since cysteinyl-LT production is increased in atherosclerosis [5] and acute coronary syndromes [6], an altered vascular sensitivity to leukotriene-induced SMC gene expression secretion may further enhance the inflammatory response. As such, targeting CysLT₁ receptors could potentially be of therapeutic interest in atherosclerosis.

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Conflict of interest None declared.

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