12- and 15-lipoxygenases in human carotid atherosclerotic lesions: Associations with cerebrovascular symptoms

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**Abstract**

Lipoxygenase (ALOX) enzymes are implicated in both pro- and anti-atherogenic processes. The aim of this study was to investigate mRNA expression of 12- and 15-lipoxygenases (ALOX12, ALOX12B, ALOX15, ALOX15B) and the atypical ALOXE3 in human carotid atherosclerotic lesions, in relation to cerebrovascular symptoms and risk factors.

The Biobank of Karolinska Endarterectomies (BiKE) collection of human carotid plaque tissue and associated clinical data was utilized (n = 132). Lesion mRNA levels were analyzed by TaqMan qPCR (n = 132) and microarray hybridization (n = 77).

Of the investigated mRNAs, only ALOX15B (15-LOX-2; epidermis-type 15-LOX) was readily detected in all plaque samples by qPCR, and thus suitable for quantitative statistical evaluation. ALOX12, ALOX12B, ALOX15 and ALOXE3 were detected with lower frequency and at lower levels, or virtually undetected.

Microarray analysis confirmed ALOX15B as the most abundant 12- or 15-lipoxygenase mRNA in carotid lesions. Comparing plaques with or without attributable cerebrovascular symptoms (amaurosis fugax, transient ischemic attack, or stroke), ALOX15B mRNA levels were higher in symptomatic than asymptomatic plaques (1.31 [1.11–1.56], n = 102; and 0.79 [0.55–1.15], n = 30, respectively; p = 0.008; mean [95% CI], arbitrary units). Multiple regression analysis confirmed symptomatic/asymptomatic status as a significant determinant of ALOX15B mRNA levels, independently of potentially confounding factors.

Immunohistochemical analyses showed abundant ALOX15B expression in macrophage-rich areas of carotid lesions, and lipidomic analyses demonstrated the presence of typical ALOX15B products in plaque tissue.

In summary, we observed associations between high ALOX15B expression in carotid lesions and a history of cerebrovascular symptoms. These findings suggest a link between ALOX15B and atherothrombotic events that merits further investigation.

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**Abbreviations:** AA, arachidonic acid; ALOX, lipoxygenase; CVD, cardiovascular disease; FA, fatty acid; HETE, hydroxy-eicosatetraenoic acid; KETE, keto-eicosatetraenoic acid; LA, linoleic acid; LT, leukotriene; qPCR, quantitative polymerase chain reaction.

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1. Introduction

Lipoxygenase (ALOX) enzymes have been implicated in both pro- and anti-atherogenic processes [1–4]. Human lipoxygenases constitute a group of 6 functional ALOX genes; 5-lipoxygenase (5-LO, ALOX5), platelet-type 12-LOX (ALOX12), reticulocyte-type 15-LOX (15-LO, 15-LOX-1, ALOX15), epidermis-type 12(R)-LOX (ALOX12B), epidermis-type 15-LOX (15-LOX-2, ALOX15B), and epidermis-type LOX3 (ALOXE3) [2,3]. Biochemical studies have however demonstrated that ALOXE3 is not a typical lipoxyge-
nase, but more correctly described as a hydroperoxide isomerase [5].

ALOX enzymes metabolize arachidonic acid (AA) and linoleic acid (LA) into oxygenated fatty acid (FA) derivatives (oxylipins) with a wide range of physiological and pathological functions such as regulation of inflammation and cell proliferation, and have therefore gained great interest with respect to e.g. inflammatory and allergic disorders, cancer, and cardiovascular disease (CVD) [1–4,6,7].

The importance of the ALOX5 pathway of leukotriene (LT) production and signalling in vascular disease was highlighted by human genetic association studies, and cardiovascular clinical trials targeting this pathway have been initiated [1,7]. “Platelet-type” ALOX12 and “reticulocyte-type” ALOX15 have been studied extensively in vitro and in animal models of CVD, but studies in human disease are scarcer [2–4]. However, the discovery of high ALOX15B expression in human carotid lesions indicated that “epidermis-type” lipooxygenases (at least this specific member) may be relevant in human atherosclerosis [8,9], and therefore further studies of 12- and 15-lipoxygenases are warranted.

We have previously characterized human carotid lesion expression of ALOX5 and other genes involved in LT synthesis and signalling, and found that enzymes of the LT pathway are increased in patients with recent symptoms of plaque instability [10]. The current aim was to expand our studies of ALOX genes in human atherosclerosis by investigating mRNA expression of 12- and 15-lipoxygenases (ALOX12, ALOX12B, ALOX15, ALOX15B) and the atypical ALOX3 in human carotid lesions, in relation to cerebrovascular symptoms and risk factors.

2. Methods

2.1. Human subjects, tissue specimens and clinical data

Human plaque tissue was obtained from the Biobank of Karolinska Endarterectomies (BiKE) study. BiKE includes asymptomatic/symptomatic patients diagnosed with >70% carotid artery stenosis (NASCET criteria) referred to the Department of Vascular Surgery for surgical treatment of severe carotid artery stenosis. Endarterectomy specimens were washed in PBS and divided longitudinally into two pieces, one of which was immediately frozen and stored at −80°C until RNA extraction and the other prepared for immunohistochemical analysis as previously described [10]. Several clinical variables were registered, including gender, clinical chemistry, medication, and last recorded symptoms of plaque instability, defined as cerebral transitory ischemic attacks, minor stroke, and/or amaurosis fugax, prior to the endarterectomy procedure. Informed consent was obtained from all subjects, and the investigation was approved by the Ethical Committee of Northern Stockholm and was in agreement with institutional guidelines and the principles set forth in the Declaration of Helsinki. A total of 132 patients were included for lesion expression analysis by quantitative PCR (qPCR), of which n = 77 were also analyzed by microarray experiments.

2.2. RNA extraction and qPCR

The frozen plaques were cut into small pieces on dry ice and transferred to FastPrep shaking tubes containing ceramic beads (Qbiogene) and an equal volume of RLT-lysis buffer and phenol, and the tissue was disrupted using FastPrep FP120 equipment (Qbiogene). RNA purification was performed using RNeasy total RNA isolation kit (Qiagen), including a DNase treatment step. RNA quantity and quality were determined using ND-1000 spectrophotometer (NanoDrop Technologies) and Agilent Bioanalyzer 2100 (Agilent Technologies) equipment. For qPCR, cDNA was synthesized from 2 μg of total RNA using random hexamer primers and SuperScript II (Invitrogen), with RNA protection by RNAsin (Promega). Quantitative TaqMan real-time PCR was performed with the ABI Prism 7700 sequence detector and software (Applied Biosystems). Samples were run for 40 cycles in duplicate, taking the mean of duplicate cycle threshold (Ct) values as measured value. A dilution series of a human multi-cell line reference cDNA (Promega) was utilized as positive control. Relative expression was determined by the ΔΔCt method using the total sample mean as calibrator [11], with normalisation to the geometric mean of multiple stably expressed transcripts (ribosomal protein, large, P0 [RPLP0] and TATA-box binding protein [TBP]) in accordance with Vandesompele [12]. The following assays-on-demand were used: ALOX12 Hs01000551_m1, ALOX15 Hs00609608_m1, ALOX15B Hs01000551_m1, ALOX3 Hs00222134_m1, RPLP0 433761F, TBP 433769F.

2.3. Microarray analysis

Biotinylated RNA was generated using 10μg of total RNA. Biotin-labeled cRNA was hybridized to Affymetrix HG-U133 plus 2.0 Genechip® arrays, stained, washed and scanned according to standard Affymetrix® protocol (www.affymetrix.com) at the BEA Karolinska core facility for expression analysis. CEL-files used in data analysis were generated with Affymetrix software, and robust multi-array average (RMA) [13] normalisation was performed as implemented in the Affymetrix Power Tools 1.10.2 package apt-probeset-summarize. Processed gene expression data were returned in a log2-scale.

2.4. Immunohistochemical analysis

Serial paraffin-embedded sections (5 μm) of human carotid atherosclerotic lesions were analyzed by immunohistochemistry after high-temperature antigen unmasking. Sections were stained with polyclonal rabbit anti-human ALOX15B (1:350; Cayman Chemical), monoclonal mouse anti-human CD68 (1:200; Leica Microsystems), monoclonal rabbit anti-human/mouse cleaved caspase-3 (Asp 175; 1:50; R&D Systems), monoclonal mouse anti-human von Willebrand Factor (VWF; 1:1000; Dakopatts), and monoclonal mouse anti-human smooth muscle α-actin (1:400; Dakopatts). Detection was performed with Mach2 Double Stain 2 and Mach3 Polymer Detection technology (BioCare Medical), using Vulcan Fast Red (BioCare Medical) and Cardassian DAB Chromogen (BioCare Medical). Rabbit IgG1 and mouse IgG1 (BioCare Medical) were utilized as negative controls. Hematoxylin (Vector Laboratories) was used for nuclear staining.

2.5. LC–MS/MS analysis of lesion oxylipins

In order to assess the presence of lipooxygenase activity in vivo, oxylipins were quantified (as pmol/g tissue) in 3 plaque tissue samples (1 asymptomatic, 2 symptomatic) using modifications of previously reported procedures [14,15]. Briefly, from plaques held on dry ice, a ~100 mg sub-sample was removed by shaving and placed into a 4 mL polypropylene cryotube on wet ice. Deuterated surrogates in 10 μL methanol were immediately introduced to sub-samples and 500 μL iced, argon-purged methanol was added. Samples were homogenized on wet ice, followed by 5-min 0°C centrifugation. Supernates were removed and diluted with de-ionized water to a 10% methanol content and extracted using Oasis HLB solid phase extraction cartridges. Analytes were eluted with methanol followed by ethyl acetate into cryotubes containing 5 μL 30% glycerol in methanol.
Extraction solvents were stripped via SpeedVac centrifugation (Heto Laboratory Equipment A/S). Residues were resuspended in 100 μL of 400 nmol/L 1-cyclohexyluriedo-3-dodecanoic acid (CUDA; Cayman Chemical) in methanol, vortexed, and filtered at 0.1 μm using Amicon Ultrafree-MC durapore PVDF filters (Millipore). Analytes were separated by reverse phase ultra performance liquid chromatography (UPLC; Waters Corp.) on a 1.7 μm Acquity BEH column as described [15]. Oxylipins were detected by negative mode electrospray ionization tandem quadrupole mass spectroscopy using published methods [15]. Ionization and fragmentation energies for the reported oxylipins were optimized for analysis on an API4000 QTrap (Applied Biosystems). Oxylipin quantities were expressed as relative composition for presentation and statistical analysis.

2.6. Statistical analysis

Data are presented as mean with 95% confidence interval (95% CI) unless otherwise specified. Differences between means were evaluated by one-way ANOVA or Student’s t-test. Non-normally distributed variables (levels of ALOX15B mRNA, triglycerides, C-reactive protein [CRP]) were log-transformed prior to parametric statistical testing. Linear regression and correlation analysis were used to evaluate relationships between continuous variables. Multiple linear regression analysis with categorical data coded as dummy variables was used to evaluate independent determinants of ALOX15B expression. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Study subjects

Detailed characteristics of the 132 study subjects are provided in Supplementary Table I. Subjects were on average 71 years of age, 78% were male, and the vast majority (circa 80–90%) were prescribed anti-platelet, anti-hypertensive and/or lipid-lowering drugs (statins). Furthermore, 77% had symptomatic carotid lesions, i.e. had recently experienced amaurosis fugax, a transient ischemic attack, or a stroke that was attributed to the lesion for which the patient underwent endarterectomy. Among symptomatic lesions, 39% were associated with stroke.

3.2. ALOX expression levels

Of the five ALOX genes analyzed by TaqMan qPCR in this study (ALOX12, ALOX12B, ALOX15, ALOX15B, ALOXE3), only ALOX15B was readily detected in all plaque samples and thus suitable for quantitative statistical evaluation (whole-sample median C<sub>t</sub> = 29.3). The other ALOX mRNAs were detected with lower frequency and at later C<sub>t</sub> values or virtually undetected, although positive control cDNA was readily amplified. For these 4 genes, the whole-sample median C<sub>t</sub> values were 36.5 for ALOX12, 37.6 for ALOX15, 39.5 for ALOXE3, and above the 40-cycle threshold for ALOX12B, whereas positive control cDNA C<sub>t</sub> values ranged from 23.9 to 26.6.

Expression levels of ALOX genes were investigated in carotid lesions by microarray analysis, including ALOX5 for reference (Fig. 1). For genes represented by multiple probesets on the microarray (ALOX5, ALOX15B, ALOXE3), the probeset giving the highest signal was considered. This analysis indicated ALOX15B as the most abundantly expressed gene second to ALOX5, as expected from the qPCR results. ALOX12, ALOX12B and ALOX15 mRNA were observed at levels approaching those of negative control genes. For ALOXE3 one of the two probesets (207708_at) gave signals well over negative control, whereas the other resulted in signals below negative control (222383_s_at; not shown). We found no apparent explanation for this discrepancy by computer-based sequence analysis.

Immunohistochemical evaluation of serial sections of carotid lesions showed abundant ALOX15B expression in macrophage (CD68)-rich lesion areas (Fig. 2A). ALOX15B was found to co-localize with CD68 and cleaved caspase-3, a marker of apoptosis, and endothelial marker VWF (Fig. 2B and C; Supplementary Fig. I).

3.3. ALOX15B clinical correlations

Associations between ALOX15B mRNA levels analyzed by qPCR and variables describing vascular events and risk factors (listed in Supplementary Table I) were investigated. ALOX15B mRNA levels were found to be significantly higher in symptomatic than asymptomatic lesions (1.31 [1.11–1.56], n = 102, versus 0.79 [0.55–1.15], n = 30; p = 0.008). Moreover, ALOX15B levels were lower in patients with diagnosed diabetes (0.87 [0.64–1.19], n = 33, versus 1.29 [1.08–1.55], n = 99; p = 0.03), and weakly negatively correlated with cholesterol concentrations (r = −0.18, p = 0.04). Although not significant, ALOX15B mRNA tended to be higher in patients on anti-hypertensive therapy (1.25 [1.05–1.48], n = 104, versus 0.83 [0.53–1.29], n = 22; p = 0.06) and lower in patients on lipid-lowering therapy (1.10 [0.92–1.31], n = 105, versus 1.57 [1.08–2.28], n = 25; p = 0.08). In multiple linear regression analysis, symptomatic status and anti-hypertensive drug treatment

![Fig. 1. Expression levels of ALOX mRNA in carotid lesions according to microarray hybridization (n = 77). The probeset giving the highest signal on average is shown for genes represented by multiple probesets on the microarray (ALOX5 204446_s_at [4 probesets], ALOX15B 206714_at [2 probesets], ALOX3 207708_at [2 probesets]), ALOX12B 207381_at, ALOX12 207206_s_at, ALOX15 207128_at). The dotted line indicates an approximate threshold for significant mRNA expression, set according to signal intensities of negative control probesets. The second ALOXE3 probeset (222383_s_at) gave signals below that of negative control probesets (not shown). Bars are shaded according to the results of TaqMan qPCR analyses; genes which were robustly expressed according to qPCR are represented by grey bars, genes that were not by white bars. Data are mean with 95% CI.](https://example.com/fig1)

### Table 1

<table>
<thead>
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<th>Independent variable</th>
<th>Dependent variable: log&lt;sub&gt;10&lt;/sub&gt; ALOX15B</th>
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<td>(Constant)</td>
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<tr>
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<tr>
<td>Diabetes</td>
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Multiple regression analysis was performed to investigate statistically independent predictors of ALOX15B mRNA expression in carotid atherosclerotic lesions, among major vascular risk factors and cerebrovascular symptomatic status.
were significant positive determinants of lesion ALOX15B mRNA levels, and diabetes and cholesterol levels were significant negative determinants, whereas statin treatment was not a significant determinant (Table 1). Levels of circulating CRP and fibrinogen were higher in patients with symptomatic compared with asymptomatic lesions (CRP: 3.23 [2.47–4.22] mg/L, n = 88, versus 1.81 [1.18–2.77] mg/L, n = 28; p = 0.03, fibrinogen: 3.93 [3.74–4.12] g/L, n = 99, versus 3.48 [3.21–3.76] g/L, n = 28; p = 0.008), but did not correlate with ALOX15B mRNA levels (r = −0.009, p = 0.92, and r = 0.09, p = 0.32, respectively).

3.4. Lesion oxylipins

Linoleate- and arachidonate-derived oxylipin profiles were determined by LC–MS/MS analysis of 3 lesion samples (Fig. 3). The included oxylipin species are listed in Supplementary Table II. In total, the linoleates represented the greater part of the included oxylipins (99%). For both substrates, the fatty acid (FA)–alcohols were found to be the major oxylipin pool, i.e. AA-derived hydroxy-eicosatetraenoic acids (HETEs) and LA-derived hydroxy-decadienoic acids (HODEs), respectively (Fig. 3A). The investigated oxylipins contained 5-lipoxygenase products such as 5-HETE, 5-KETE, and LTB4, and 15-lipoxygenase products such as 15-HETE, 15-KETE, and 8,15-DiHETE. As shown in Fig. 3B, the abundance of the FA–alcohols 15-HETE and 5-HETE potentially produced by ALOX15B and ALOX5, respectively, was significantly greater than that of typical FA–alcohol auto-oxidation products (8-HETE, 9-HETE and 11-HETE).

4. Discussion

This study of patients with advanced carotid atherosclerosis provides two novel pieces of information. First, our findings indicate that ALOX15B (15-LOX-2) is the only abundantly expressed gene in human carotid lesions among the four 12- and 15-lipoxygenases ALOX12, ALOX12B, ALOX15, ALOX15B and the atypical ALOXE3. Second, that ALOX15B mRNA expression is significantly higher in symptomatic compared with asymptomatic lesions, independently of potentially confounding factors.

Our data show that ALOX15B is the most abundant lipoxygenase mRNA in human carotid lesions, second to ALOX5 (Fig. 1). These findings complement and extend previous reports [8–10,16]. Previous studies have reported high ALOX15B protein expression in human carotid lesions, in macrophage-rich areas [8,9], findings that we confirmed in the current study (Fig. 2A). Interestingly, our results indicate that ALOX15B is expressed by apoptotic macrophage and by endothelial cells of intra-plaque vessels (Fig. 2B and C; Supplementary Fig. I). We and others have previously demonstrated abundant ALOX5 protein expression in human lesions [10,16]. Our current results agree with the previous finding that while ALOX5 expression is abundant in human lesions, ALOX15 (15-LOX-1) is undetectable or found only at low levels [16]. However, an earlier study of aortic samples obtained post mortem or from organ donors reported ALOX15 expression in aortic lesions, but did not detect ALOX5 (by in situ hybridization) [17]. Of note, the microarray result in Fig. 1 that seemingly suggests presence of ALOXE3 mRNA in plaque tissue should be interpreted with great caution, since one of two ALOXE3 probesets gave signals below
those of negative controls, and since ALOXE3 was barely detectable by qPCR.

A main finding of the current study was that ALOX15B mRNA is increased in symptomatic compared with asymptomatic lesions. Additionally, we found that diabetes, cholesterol levels (but not lipid-lowering therapy), and anti-hypertensive treatment were statistically independent determinants of ALOX15B expression in the current model (negative or positive; Table 1). The fact that these variables describing major vascular risk factors (or therapy thereof) statistically influenced ALOX15B expression in different directions may appear contra-intuitive, and the underlying explanation is not obvious. Nevertheless, our results demonstrate that ALOX15B expression is higher in symptomatic than asymptomatic carotid lesions, irrespective of potentially confounding vascular risk factors and related drug treatment. We did not observe any significant differences in ALOX15B mRNA levels according to the time elapsed between the latest symptom and surgical intervention (<1 month, 1–3 months, or >3 months; not shown) which has been used as a parameter reflecting plaque stability/instability [10,18].

In addition to originally cloned full-length ALOX15B (NM_0011411.2) [19], several splice variants have been described, foremost in studies of prostate epithelial cells [9,20–22]. The majority of these alternative transcripts, including those two currently found in the curated RefSeq database (NM_001039130.1 or NM_001039131.1), do not contain exon 9 that constitutes part of the enzyme’s active site according to structural modelling, and the lack of which leads to reduced specific activity [20–22]. We utilized a TaqMan assay targeting exons 8–9 of ALOX15B and hence alternative transcript variants lacking the catalytically important potential mechanism(s) of ALOX15B in this context. The main substrate of ALOX15B is considered to be AA and its main enzymatic products of both 5- and 15-lipoxygenase activity are present to a similar degree as those of the most abundant lesion lipoxygenase ALOX5 (Fig. 3). The potential ALOX15B and ALOX5 products 15-HETE and 5-HETE were significantly greater than analogous typical auto-oxidation products (8-HETE, 9-HETE, and 11-HETE), and typically enzymatic products of both 5- and 15-lipoxygenase activity were detected, at similar levels (LTB4 and 8,15-DIHETE, respectively). These data give support for a metabolic effect of ALOX15B in atherosclerotic lesions.

In summary, this study identifies associations between high ALOX15B expression in carotid lesions and cerebrovascular symptoms, independently of potentially confounding clinical factors. These associations provide circumstantial evidence for a role of ALOX15B with respect to atherothrombotic events that merits further investigation.

Disclosures

None.

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Fig. 3. Oxylipin profiles of carotid lesions (n = 3) analyzed by LC-MS/MS (relative composition). A complete list of oxylipins included in the analysis is provided in Supplementary Table II. Panel (A) Oxylipin class profiles for linoleate (LA)- and arachidonate (AA)-derived products. It should be noted that data presented in Panel (A) is dependent on the number of compounds included for each substrate and oxylipin class, wherefore direct comparisons may be inappropriate and are to be interpreted with caution. Panel (B) Specific AA-derived oxylipins of interest. The potential ALOX15B and ALOX5 FA–alcohol products 15-HETE and 5-HETE were greater than the typically non-enzymatic FA-Alcohols 8-HETE, 9-HETE, and 11-HETE. (*** p < 0.001; one-way ANOVA with Bonferroni post hoc test). Data are mean with 95% CI.
Appendix A. Supplementary data


References