Expression of 5-lipoxygenase and leukotriene A₄ hydrolyase in human atherosclerotic lesions correlates with symptoms of plaque instability


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Leukotrienes (LTs) are a group of proinflammatory lipid mediators that are implicated in the pathogenesis and progression of atherosclerosis. Here we report that mRNA levels for the three key proteins in LTB₄ biosynthesis, namely 5-lipoxygenase (5-LO), 5-LO-activating protein (FLAP), and LTA₄ hydroxylase (LTA₄H), are significantly increased in human atherosclerotic plaque (n = 72) as compared with healthy controls (n = 6). Neither LTC₄ synthase nor any of the LT receptors exhibits significantly increased mRNA levels. Immunohistochemical staining revealed abundant expression of 5-LO, FLAP, and LTA₄H protein, colocalizing in macrophages of intimal lesions. Human lesion tissue converts arachidonic acid into significant amounts of LTB₄ and a selective, tight-binding LTA₄H inhibitor can block this activity. Furthermore, expression of 5-LO and LTA₄H, but not FLAP, is increased in patients with recent or ongoing symptoms of plaque instability, and medication with a selective inhibitor of LTA₄H can block LTB₄ biosynthesis in plaque tissue, thus identifying LTA₄H as a potential target for development of drugs for prevention and treatment of atherosclerosis and acute atherothrombotic events.

Results
Expression of 5LO, FLAP, LTA4H, and LTC4S mRNA in Human Carotid Plaque and Mouse Atherosclerotic Aorta. In the human carotid atherosclerotic lesions, the transcript levels of 5-LO, FLAP, and LTA4H were high, corresponding to a 7.5-fold (7.5 ± 4.1, P < 0.001), 2.7-fold (2.7 ± 1.3, P = 0.003), and nearly 2-fold (1.9 ± 1.0, P = 0.03) increase relative to normal iliac arteries, respectively (Fig. L1). In contrast, the levels of LTC4S mRNA were not significantly different from controls.

In the ApoE(−/−) mouse aorta, 5-LO and LTA4H mRNA levels were essentially unaltered, whereas FLAP and LTC4S mRNA showed a tendency to increase relative to wild-type C57BL/6J mice (Fig. 1B). In ApoE(−/−) × LDLR(−/−) mice, the expression pattern of the enzymes was essentially the same as in ApoE(−/−) mice, except that LTA4H mRNA levels were significantly increased (1.7 ± 0.3, P = 0.01) (Fig. 1C).

Expression of 5-LO, FLAP, and LTA4H Protein in Human and Mouse Atherosclerotic Lesions. Staining of human carotid plaque demonstrated prominent expression of 5-LO, FLAP, and LTA4H in intimal lesion areas that also stained positively for human macrophage marker CD163 (Fig. 2). The distribution of 5-LO, LTA4H, and FLAP was comparable to that of human lesions.

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Figs. 1 and 2 are reprinted, with permission, from PNAS (17) and J. Am. Coll. Cardiol. (18). Abbreviations: LT, leukotriene; CysLT, cysteinyl-LT receptor; 5-LO, 5-lipoxygenase; LTA₄H, LTA₄ hydroxylase; FLAP, 5-LO-activating protein; LTC₄S, LTC₄ synthase; LDLR, low-density lipoprotein receptor; ApoE, apolipoprotein E; EIA, enzyme immunoassay.

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The human and mouse data, respectively. Data are presented as mean ± SD.

**Expression of 5-LO, FLAP, LTA4H, and LTC4S mRNA in human carotid plaque and mouse atherosclerotic aorta.** Expression of 5-LO, FLAP, LTA4H, and LTC4S mRNA in human carotid plaque (n = 72) and mouse atherosclerotic aorta from ApoE (−/−) mice (n = 8) (A), and ApoE (−/−) × LDLR (−/−) mice (n = 5) (B) were determined by real-time quantitative RT-PCR and compared with normal human arteries (n = 6) and with the age-matched C57BL/6J strain (n = 8) for the human and mouse data, respectively. Data are presented as mean ± SD. * P < 0.05; ** P < 0.01; *** P < 0.001.

FLAP, and LTA4H were very similar, indicating a high degree of coexpression in cells of the atherosclerotic lesions, particularly macrophages.

Mouse aortic plaques displayed abundant expression of LTA4H within the intimal lesion area, with distribution similar to CD68-positive macrophages (Fig. 3A and B). However, serial sections stained for LTA4H and 5-LO revealed segregated distribution of these two enzymes. Thus, LTA4H staining was exclusively located in tunica intima, whereas the majority of 5-LO-positive cells were present in the adventitia (Fig. 3D and E). Immunofluorescence double staining demonstrated accumulation of LTA4H protein in the subendothelial area of intimal lesions, partly colocalized with CD68, and some in cells that were in close proximity to CD68+ macrophages (Fig. 3F–I). Double staining of the same tissue for 5-LO and CD68 revealed localization of 5-LO protein with CD68 in tunica adventitia but barely in intimal lesions (Fig. 3K–N). The antibodies against human FLAP did not allow a reliable immunohistochemical localization of the corresponding mouse protein.

**Biosynthesis of LTB4 and Effects of an LTA4H Inhibitor.** Homogenates of human plaque tissue incubated with arachidonic acid produced significant amounts of LTB4 (2.2 ± 0.5 ng/g of tissue), as assessed by reverse-phase HPLC coupled to enzyme immunoassay (EIA), and this biosynthesis could be reduced by ~80% after preincubation with 10 μM selective, tight-binding, amino-hydroxy acid inhibitor of LTA4H (Fig. 4). Incubations of homogenates of mouse plaque with arachidonic acid yielded similar results (data not shown).

**Correlation of 5-LO, FLAP, and LTA4H Expression Levels with Clinical Parameters.** The mRNA levels of 5-LO, FLAP, and LTA4H in the atherosclerotic carotid plaques were correlated with the occurrence of the patients’ clinical symptoms. Interestingly, the mRNA levels of both 5-LO and LTA4H were significantly higher in patients reporting recent clinical events of transitory cerebral ischemic attacks, minor stroke, and/or amaurosis fugax (Fig. 5A). None of the mRNA levels was affected by gender, plasma cholesterol level, or pharmacological treatment with statins or angiotensi-converting enzyme inhibitors (data not shown). However, the mRNA levels of FLAP were significantly increased in plaques from patients on treatment with warfarin (Fig. 5B), whereas samples from patients taking aspirin showed a tendency of decreased FLAP mRNA levels (data not shown).

**Expression of LT Receptors in Human and Mouse Atherosclerotic Lesions.** In human carotid plaque, the expression of the receptors for LTB4 or CysLTs was not significantly changed compared with control tissues from the iliac artery (Fig. 6A). Analysis of mouse atherosclerotic aorta for CysLT1 mRNA levels detected a 2.5-fold (2.5 ± 1.0, P = 0.002) increase in ApoE (−/−) mice and a 3-fold (3.3 ± 0.8, P < 0.001) increase in ApoE (−/−) × LDLR (−/−) mice compared with normal control mice (Fig. 6B and C). In addition, a 3-fold (3.4 ± 2.1, P = 0.007) increase of CysLT2 mRNA levels was observed in ApoE (−/−) mice (Fig. 6B). Immunohistochemical staining detected CysLT1 protein within the mouse atherosclerotic lesions, with distribution similar to CD68+ macrophages (Fig. 7A and B). Immunofluorescence double staining confirmed the colocalization of CysLT1 protein with CD68 in intimal lesions (Fig. 7E–H).
During the past few years, the focus of the LT research field has expanded from allergic and inflammatory diseases of the respiratory tract to another disease characterized by chronic inflammation, namely, atherosclerosis (14). Early data in the literature associated LT production with ischemic heart disease (18), but for long 5-LO was generally believed to be absent from advanced human atherosclerotic lesions (19). Three almost simultaneous studies sparked new interest in this area; first, the demonstration that 5-LO is abundantly expressed in human atherosclerotic lesions and correlates with disease stage (8); second, the observation that a selective BLT1 antagonist could protect against atherogenesis in two mouse models of atherosclerosis (10); and, third, the key report that deletion of the 5-LO gene affords protection in atherosclerosis-prone mice (9). These reports were followed by genetic studies identifying promoter variants of 5-LO and the FLAP gene as significant risk factors for development of atherosclerosis and myocardial infarction in man (12, 13), which in turn led to the exciting prospect of anti-LTs as potential therapeutics for cardiovascular disease (11). However, studies conducted thus far have generated information that does not conform to a consistent picture. Studies have usually focused on a single enzyme or receptor using human tissues or a specific mouse model of atherosclerosis. Here we exploited the large Biobank of Karolinska Endarterectomies collection of human atherosclerotic vascular tissues obtained from patients undergoing carotid endarterectomy as well as plaque tissues from two mouse models of atherosclerosis to generate a comprehensive expression profile of key enzymes and receptors of the LT cascade in humans and mice.

Expression of 5-LO, FLAP, and LTA4H Is Increased and Colocalized in Human Atherosclerotic Lesions. Analysis of atherosclerotic tissues from 72 patients revealed a striking increase in the expression of 5-LO mRNA, confirming previous data on human atherosclerotic specimens (8). In addition, we found significant increases in the mRNA levels of FLAP and LTA4H, thus completing the triplet of key proteins required for LTB4 biosynthesis (Fig. 1) and corroborating the notion that this specific LT is critically involved in human atherosclerosis (10, 13, 16, 17, 20). In contrast, neither LTC4S nor any of the LT receptors displayed significantly elevated levels of mRNA. Furthermore, 5-LO, FLAP, and LTA4H proteins colocalize in intimal lesions (Fig. 2), particularly in macrophages, presumably facilitating efficient enzyme coupling and LTB4 synthesis. Moreover, the lack of symmetrical increases of all LT cascade proteins suggests the existence of regulatory mechanisms, yet to be identified, which influence their expression levels within the atherosclerotic lesions, as very recently demonstrated for 5-LO expression in unstable plaque (21).

Expression of 5-LO and LTA4H mRNA Correlates with Recent or Ongoing Atherothrombotic Events. We found a significant correlation between the levels of 5-LO and LTA4H mRNA and recent or ongoing symptoms of plaque instability (Fig. 5). Such events are caused by atherothrombosis in unstable plaques, which eventually go through a healing process (22). Presumably, the time interval between the last recorded symptom or the surgical procedure represents unstable plaques at different stages of healing/instability. Therefore, the data point to an association between expression of 5-LO and LTA4H and plaque...
instability. Together with genetic data identifying 5-LO as a risk factor for atherosclerosis (12), it is tempting to speculate that LTs may be involved in driving local acute inflammatory processes that precede and precipitate an acute thrombotic event. Hence, these findings are potentially of clinical significance and suggest that anti-LTs may be useful, not only in reducing atherosogenesis, but also in the prevention and treatment of acute atherothrombotic complications.

Treatment with Warfarin Correlates with Increased Expression of FLAP mRNA in Atherosclerotic Plaques. We were surprised to find that levels of FLAP mRNA were significantly higher in patients taking warfarin (Fig. 5B). This drug inhibits blood clotting by means of reduced liver production of several vitamin K-dependent coagulation factors (II, VII, IX, and X), and a mechanism for its effect on FLAP mRNA expression is not obvious. Warfarin medication is usually withdrawn 2–3 days before endarterectomy and substituted with other drugs, which may also influence FLAP gene expression. In addition, we noted a tendency of decreased FLAP mRNA levels in patients taking aspirin, a drug that is seldom, if ever, combined with warfarin treatment.

Differences in mRNA and Protein Expression Between Human and Murine Lesions. Although several lines of evidence indicate a role for LTs in particular LTBs, in atherosclerosis, the data presented thus far are not fully consistent. For instance, deletion of the 5-LO gene in mice with an LDLR-deficient genetic background effectively protected against atherosclerosis (9), whereas a more extensive study failed to detect any similar effects but rather a protection against aortic aneurysm (15). Because 5-LO deletion leads to a complete loss of all LTs, the latter study is difficult to reconcile with data demonstrating that pharmacological antagonism or genetic deletion of BLT1 in mice also affords protection against atherosclerosis (10, 16, 17). We found that expression of 5-LO mRNA was not significantly up-regulated in ApoE (−/−) or ApoE (−/−) × LDLR (−/−) mice, and the distribution of 5-LO and LTA4H proteins was segregated (Fig. 3). Furthermore, CysLT1 and CysLT2 expression was strongly up-regulated in the mouse models (Figs. 6 and 7). These findings are consistent with the report by Zhao et al. (15), in which the atherosclerosis-prone mouse strains displayed expression of 5-LO in the adventitia (rather than intimal lesions).
and a profound increase in CysLT₁ message levels. Yet abolition of LT production in these mice failed to protect against atherosclerosis (15). Hence, it appears that the expression patterns are different in mouse models and patients with respect to LT-dependent atherogenesis, which in turn has considerable pharmacological implications. One must also keep in mind that mouse models reflect early phases of atherosclerosis, whereas human surgical specimens are end-stage lesions causing clinical symptoms after years of progression. The spectrum of factors operating in the destabilization of such advanced lesions and subsequent thrombus formation is likely to differ significantly from that promoting initiation of the disease process. LTA₄H, a Potential Target for Treatment of Atherosclerosis and Acute Thrombotic Complications. Human plaque tissue displayed increased expression of 5-LO, FLAP, and LTA₄H colocalizing in intimal lesions, and indeed we found that it also converts arachidonic acid into LTB₄. Because LTA₄H catalyzes the committed step in LTB₄ biosynthesis, we tested the effects of an LTA₄H inhibitor and found that it effectively suppresses the synthesis of LTB₄ (Fig. 4). It is interesting to note that inhibition of LTA₄H preserves LTA₂ synthesis, which in turn may promote formation of anti-inflammatory lipoxins and related molecules (23). Hence, LTA₄H appears to be a promising target for development of drugs for prevention and treatment of atherosclerosis and its associated thrombotic complications.

Methods

Animals. Male ApoE (−/−) mice and ApoE (−/−) × LDLR (−/−) mice on the C57BL/6J background were obtained from Malling Beck (Ry, Denmark) after they were weaned. The ApoE (−/−) × LDLR (−/−) mice were fed a Western-type diet containing cornstarch, glucose, sucrose, cocoa butter, cellulose, minerals, vitamin mix, 0.15% cholesterol, and 21% total fat (wt/wt) for 8 months (24). The ApoE (−/−) mice were fed standard chow for 25 weeks. Age-matched C57BL/6J mice fed standard chow served as controls. Mice were killed at 8 months [for ApoE (−/−) × LDLR (−/−) mice] or 25 weeks [for ApoE (−/−) mice], and the proximal part of the aorta was collected, snap-frozen in liquid nitrogen, and stored at −80°C. The housing and care of the animals and all of the animal procedures used in this study were in accordance with national guidelines and approved by the Stockholm North Ethical Committee on Animal Experiments.

Human Tissue Specimens and Clinical Data. Human plaque tissue was obtained from the Biobank of Karolinska Endarterectomies study. Samples of atherosclerotic tissue collected from patients undergoing carotid endarterectomy were washed in PBS and divided longitudinally into two pieces. One was immediately frozen and stored at −80°C until RNA extraction, and the other was mounted in OCT medium for cryosectioning, snap-frozen on dry ice, and stored at −80°C until immunohistochemical analysis. Control tissue was obtained from iliac arteries of organ donors. The adventitia and outer media were removed by dissection before freezing. Several clinical variables were registered for each patient included in the Biobank of Karolinska Endarterectomies, such as gender, plasma cholesterol level, medication, and last recorded symptoms of plaque instability, defined as cerebral transitory ischemic attacks, minor stroke, and/or amaurosis fugax, before 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.

RNA Extraction and Quantitative Real-Time PCR. Extraction of total RNA was performed by using the RNeasy Mini kit (Qiagen, Valencia, CA) including an on-column DNase digestion step. RNA concentration and quality were assessed by being analyzed by a Bioanalyzer capillary electrophoresis system (Agilent Technology). CDNA was synthesized from total RNA by using SuperScript II reverse transcriptase (Invitrogen), and real-time PCR was performed in a TaqMan 7700 instrument (Applied Biosystems). Normalizations were made to cyclophilin A in the human and β-actin in the murine experiments. The primer/probe pairs for human FLAP and mouse 5-LO were obtained by Assay-by-Design (Applied Biosystems). Primers were as follows: 5-LO forward primer sequence, GCTTATCCTGAGTTGCGCTTTTT; 5-LO reverse primer sequence, AGCCCTCTCGACCCCTTG; 5-LO reporter sequence, ACAAGGCCTAATGGCACCCG; FLAP forward primer sequence, GCCCTTTGCAGCGGTCTACAC; FLAP reverse primer sequence, AGAGCAACAGCAGGAAAGTG; FLAP reporter sequence, CTGCCAACAGCAACTG. The following primer/probe pairs were obtained by Assay-on-Demand (Applied Biosystems): human 5-LO, LTA₄H, LTC₄S, CysLT₁, CysLT₂, BLT₁, and BLT₂ with assay IDs Hs00167536_m1, Hs00168505_m1, Hs00168529_m1, Hs00272624_s1, Hs00252658_s1, Hs00175124_m1, and Hs01594782_m1, respectively; mouse FLAP, LTA₄H, LTC₄S, CysLT₁, CysLT₂, BLT₁, and BLT₂ with assay IDs Mm00802100_m1, Mm00521826_m1, Mm00521864_m1, Mm00445433_m1, Mm00521826_m1, Mm00521864_m1, Mm00445433_m1, Mm00521826_m1, Mm00521864_m1, Mm00445433_m1, Mm00521826_m1, Mm00521864_m1, Mm00445433_m1, Mm00521826_m1, Mm00521864_m1, Mm00445433_m1, Mm00521826_m1, Mm00521864_m1, Mm00445433_m1, Mm00521826_m1, Mm00521864_m1, Mm00445433_m1, Mm00521826_m1, Mm00521864_m1, Mm00445433_m1,
respectively.

**Immunohistochemistry.** Cryostat sections (10 μm) of human carotid plaque or mouse aorta were fixed in acetone for 10 min, air-dried, and stained by using biotin–avidin–immunoperoxidase technology (20). Rabbit polyclonal antibodies against human LTA4H (prepared in-house), human 5-LO (a gift from Olof Rådmark; Karolinska Institutet, Stockholm), human FLAP (a gift from Jilly Evans; Merck Frosst Labs, Pointe Claire, PQ, Canada), human CysLT1 receptor were combined with rat anti-mouse CD68 serum, primary antibodies from rabbit against LTA4H, 5-LO, or double staining, after preincubation with 10% normal goat serum. Mouse monoclonal antibodies against human CD163 (DakoCytomation) were detected with biotinylated IgG (Vector Laboratories). Mouse monoclonal antibodies against human CD68 (DakoCytomation) were detected with biotinylated rabbit anti-mouse IgG (Vector Laboratories), and rat polyclonal anti-mouse CD68 was detected with biotinylated rabbit anti-rat IgG (Vector Laboratories). All antibodies were used at optimal dilutions determined by titration on mouse spleen and human tonsils, respectively. For immunofluorescence double staining, after preincubation with 10% normal goat serum, primary antibodies from rabbit against LTA4H, 5-LO, or CysLT1 receptor were combined with rat anti-mouse CD68 (1:10,000), applied on sections overnight at 4°C, rinsed, and incubated with Cy3-conjugated goat anti-rabbit-IgG (1:400; Jackson ImmunoResearch) and FITC-conjugated goat anti-rat-IgG (1:200; Jackson ImmunoResearch) at room temperature for 1 h. After staining DNA with DAPI, slides were mounted with an antifading mounting medium (Vector Laboratories) and observed under epifluorescence. The specificity of primary antibodies was assessed by neutralizing the primary antibody with a 20-fold excess of blocking protein or peptide at room temperature for 1 h before use.

**Reverse-Phase HPLC Coupled to EIA.** Human carotid plaques were collected immediately after endarterectomy, dissected, weighed, homogenized in 0.1 M PBS containing protease inhibitor mixture (Roche), and sonicated three times for 10 s on ice. Homogenates were centrifuged at 10,000 × g for 10 min at 4°C, and the resulting tissue extracts were preincubated in the presence or absence of 10 μM aminohydric acid, synthesized as described (25), for 30 min at room temperature. Samples were incubated with 2 mM ATP, 2 mM CaCl2, and 80 μM arachidonic acid for 10 min and quenched with an equal volume of methanol. After acidification to pH 3–4, samples were purified by solid-phase extraction (Supelclean LC18, Supelco) and analyzed by reverse-phase HPLC. The column (Nova-Pak C18, Waters) was eluted with acetonitrile/methanol/water/acetic acid (30:35:35:0.01 by volume) at 1.0 ml/min, and absorbance was monitored at 270 nm. Fractions corresponding to the retention time of an LTβR standard (Cayman Chemical) were collected, dried under nitrogen, and resuspended with EIA buffer. Levels of LTβR were determined in triplicate assays with an LTβR ELISA kit (Cayman Chemical) by using dilutions within the linear portion of the standard curve.

**Statistical Analysis.** Data are presented as mean ± SD. Differences between the means were evaluated by one-way ANOVA, Student’s t test, or the Mann–Whitney test when appropriate. A value of P < 0.05 was considered statistically significant.

**Note.** During the preparation of this article, it was reported that the LTA4H gene, alone or in combination with the FLAP gene, constitutes a significant risk factor for myocardial infarction in several human populations (26), thus further corroborating the results and conclusions of the present study.

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