Differential Leukotriene Constrictor Responses in Human Atherosclerotic Coronary Arteries

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Background—Leukotrienes are a class of biologically active lipids that have potent effects on the heart. To assess their role in coronary artery disease, we compared the contractile responses of leukotriene C₄ (LTC₄) and leukotriene D₄ (LTD₄) and their binding activity in both atherosclerotic and nonatherosclerotic human coronary arteries. We also studied expression of the enzymes that control their formation to understand how the 5-lipoxygenase (5-LO) pathway is activated in the coronary arteries.

Methods and Results—The capacity of leukotrienes to affect coronary vessel tone and the influence of atherosclerosis was tested in organ baths. Leukotriene receptors were examined by autoradiography, and antibody binding to the various enzymes responsible for their formation was assessed by use of immunocytochemistry. Nonatherosclerotic coronary artery ring segments were unresponsive to LTC₄ and LTD₄. In contrast, LTC₄ and LTD₄ induced concentration-dependent contractions in atherosclerotic coronary arteries. Specific [³H]-LTC₄ but not LTD₄ binding to atherosclerotic coronary artery was evident, with no evidence of specific binding of [³H]-leukotrienes to nonatherosclerotic coronary artery. High-resolution autoradiography identified specific [³H]-LTC₄ binding sites to smooth muscle cell and to regions of intimal proliferation and plaque. Cells showing positive antibody binding to 5-LO, FLAP (5-lipoxygenase activating protein), and leukotriene A₄ hydrolase were also present in the coronary arteries and had a similar distribution to macrophages.

Conclusions—Atherosclerosis is associated with a specific leukotriene receptor(s) capable of inducing hyperreactivity of human epicardial coronary arteries in response to LTC₄ and LTD₄. (Circulation. 1998;97:2406-2413.)

Key Words: atherosclerosis ■ leukotrienes ■ coronary disease

Coronary atherosclerosis is a complex and dynamic multifactorial disease that depends on the exchange of biochemical messages by resident cells (endothelial and smooth muscle cells) and infiltrating leukocytes that regulate functions critical to lesion initiation and progression and to the clinical manifestations of coronary artery disease.¹ The clinical manifestations include stable or unstable angina, acute myocardial infarction, and sudden cardiac death. An episodic increase in vasomotor tone of epicardial coronary arteries is an important pathological component of a number of these ischemic cardiac syndromes; however, its causes remain unclear.²⁻⁴ Infiltrating leukocytes provide a source of a number of vasoactive mediators with the potential to produce these effects.

Leukotrienes are a class of biologically active lipids, synthesized and released from leukocytes, that have a variety of proinflammatory effects.⁵⁻⁶ The synthetic pathway for leukotrienes is initiated by the release of arachidonic acid from the cell membrane by phospholipase A₂, followed by its conversion to leukotriene A₄ (LTA₄) by the enzyme 5-LO and its activating protein, FLAP.⁷⁻⁸ LTA₄ is either converted to LTB₄ by the enzyme LTA₄ hydrolase⁹⁻¹⁰ or is conjugated with glutathione to form the cysteinyl leukotriene LTC₄.¹¹ The cysteinyl leukotrienes include LTC₄ and its metabolites, LTD₄¹² and LTE₄.¹³ There is a growing body of evidence suggesting a putative role of leukotrienes in coronary heart disease.¹⁴ In particular, the cysteinyl leukotrienes are potent vasoconstrictors of coronary arteries of several species¹⁵⁻¹⁷ and have been shown to be associated with myocardial ischemic events, such as in experimentally induced myocardial infarction¹⁸ and in “cardiac anaphylaxis.”¹⁹ In addition, inhibitors of 5-LO²⁰ and cysteinyl leukotriene receptor antagonists²¹ are effective in reducing infarct size and reperfusion-induced arrhythmias in animal models of experimental ischemia. These studies, together with recent clinical evidence of an increased production of cysteinyl leukotrienes in patients with coronary artery disease,²²,²³ implicate involvement of leukotrienes in coronary heart disease. Therefore, to assess their role in coronary artery disease, we compared the contractile responses of LTC₄ and LTD₄ and their binding activity in both atherosclerotic and nonatherosclerotic human coronary arteries. We also studied expression of the enzymes that control their formation to understand how the 5-LO pathway is activated in human coronary arteries.
Methods

Materials

LTC_4 and LTD_4 were purchased from Cascade Biochem. [^3H]-LTC_4, [^3H]-LTD_4, nuclear emulsion (LM-I), and hyperfilm were purchased from Amersham International. Acivicin, 1-cysteine, indomethacin, 3,3-diaminobenzine tetrahydrochloride, and Tris HCL were purchased from Sigma Chemicals. Rabbit polyclonal antisera to purified 5-LO, FLAP amino acids 41 to 52, and LTA_4 hydrolase were kindly provided by Dr Jilly Evans (Merck Frosst, Pointe Claire-Doval, Canada). Other chemicals were of reagent grade and were obtained from BDH Chemicals.

Patient Details

Human epicardial coronary arteries were removed from explanted hearts of 44 patients at the time of cardiac transplantation. Informed consent was obtained from each patient, and the study was approved by the Harefield Hospital Ethical Committee. Atherosclerotic arteries were obtained from 22 patients with previously diagnosed IHD who had obstructive coronary lesions by angiography (mean age, 57 years; range, 49 to 63). Nonatherosclerotic arteries were obtained from 22 patients undergoing transplantation for reasons other than IHD (mean age, 30 years; range, 4 to 53). The latter group comprised 18 patients with DCM, 3 with congenital heart defects, and 1 with cystic fibrosis. These arteries were free from atheroma on microscopic and histological examination, although they occasionally showed evidence of mild intimal proliferation.

Organ Bath Experiments

LTC_4 and LTD_4 Responses

Left anterior descending and right coronary arteries from both proximal and distal regions of the vessels were dissected free from the surrounding myocardium, cut into ring segments, and mounted for isometric tension recording as described previously. In some experiments, ring segments of atherosclerotic arteries were incubated with acivicin (50 μmol/L), an irreversible γ-glutamyl transpeptidase inhibitor that prevents the metabolism of LTC_4 to LTD_4. In another group of atherosclerotic coronary artery segments, concentration-response curves were performed in the presence of 1-cysteine (3 mmol/L) to block the metabolism of LTD_4 to LTE_4.

Endothelial Modulation of LTD_4 Responses

Because LTD_4 can stimulate release of endothelium-derived nitric oxide, a separate series of experiments was conducted to investigate whether LTD_4 responses could be affected by the endothelium. Vessels were incubated with indomethacin (10 μmol/L) to block the synthesis of relaxing prostaglandins before the addition of LTD_4. In another group of experiments, vessels were preconstricted with the thromboxane mimetic U46619 (1 to 3 mmol/L) or prostaglandin F_2α (1 mmol/L), whereas in other vessels the endothelium was removed before the tissues were challenged with LTD_4. Removal of the endothelium was confirmed by the absence of relaxation to the endothelium-dependent relaxation factor substance P.

In Vitro Receptor Autoradiography

Epicardial coronary arteries were obtained from six patients undergoing heart transplantation (three DCM and three IHD) and snap-frozen in liquid nitrogen. Tritiated leukotriene binding sites in atherosclerotic and nonatherosclerotic coronary arteries were localized with the use of in vitro receptor autoradiography. The optimum incubation time (association experiments) and wash times (dissociation experiments) had been determined previously. Saturation studies were performed on slide-mounted sections of both vessel types that were initially preincubated in 50 mmol/L Tris HCl buffer, pH 7.4, for 15 minutes at 4°C to reduce levels of endogenous leukotrienes. Slides were then incubated in buffer containing 5 mmol/L CaCl_2, 0.05 mmol/L acivicin, and 20 mmol/L L-cysteine in the presence of 0.1 to 3.0 mmol/L [^3H]-LTC_4 or [^3H]-LTD_4 (specific activity, 154 Ci/mmol) for 60 minutes at 4°C. Acivicin was used to prevent the metabolism of LTC_4 to LTD_4 during the incubation, and L-cysteine prevented the metabolism of LTD_4 to LTE_4. The degree of non-specific binding was established by incubating alternate sections in the presence of 1 μmol/L unlabelled LTC_4 and LTD_4. After incubation, sections were washed twice for 5 minutes in buffer at 4°C, dipped in cold (4°C) distilled water, and dried in a stream of cold air. Low- and high-resolution autoradiography was performed as described previously by exposing incubated sections to hyperfilm for 5 weeks and apposing sections to coverslips coated with emulsion for 6 weeks in lightproof boxes at 4°C, respectively. Estimation of [^3H]-LTC_4 and [^3H]-LTD_4 binding was performed by wiping off tissue sections from the microscope slides with Nucwipes (National Diagnostic), which were then placed in Ultragold scintillant (4.5 mL) and counted for tritium as described previously.

Immunocytochemistry of 5-LO, FLAP, and LTA_4 Hydrolase

The left anterior descending arteries from eight hearts were obtained from patients undergoing transplantation (five DCM and three IHD). Frozen sections 6-μm thick were cut, and rabbit anti-5-LO (diluted 1:400), rabbit anti-FLAP (diluted 1:300), rabbit anti-LTA_4 hydrolase (diluted 1:1200), or mouse monoclonal anti-CD68 (diluted 1:1000) was applied to the sections, which were incubated for 1 hour. Tissues were then stained according to the manufacturer’s instructions.

Data Analysis

Contractions were measured as a percentage of the maximal isometric contraction to 90 mmol/L KCl. The E_max value refers to the maximum response at the highest dose of leukotriene, and the EC_{50} value for each concentration-effect curve was obtained by linear regression analysis of data points in grams or percentage of KCl. These values were transformed into pD_2 values (−log EC_{50}). All results are shown as mean±SEM, and in all experiments, n equals the number of patients from whom the vessels were obtained. Differences between leukotrienes were compared by unpaired Student’s t test. Comparisons between control and experimental groups were made by ANOVA followed by a Bonferroni correction. A value of P<.05 was considered a statistical difference.

Results

Effect of LTC_4 and LTD_4 on Vasomotor Tone

Nonatherosclerotic coronary artery ring segments were unresponsive to LTC_4 and LTD_4 from each of the three nonatherosclerotic groups (Figure 1; n=8). In contrast, LTC_4 and LTD_4 induced concentration-dependent contractions in atherosclerotic coronary arteries (Figure 1; n=11). The potency (EC_{50}) and maximum response (E_max) to LTC_4 were 11.1 nmol/L (95% CI, 9.4 to 13.0) and 62±8.4%, respectively, and to LTD_4 were 7.0 nmol/L (95% CI, 1.3 to 36) and 32±6.5%, respectively (P<.05, E_max for LTC_4 versus LTD_4). The degree of contraction of the atherosclerotic vessels induced by LTC_4 and LTD_4 after pretreatment with indomethacin was unchanged, indicating that constricting prostaglandins such as thromboxane A_2 were not involved (data not shown). Furthermore, responses to each leukotriene were prolonged (usually 20 to 30 minutes for each concentration response to plateau) and difficult to wash out.
Vessel segments treated with vehicle (MeOH:H₂O:AcOH) to control for the solvent that the leukotrienes were dissolved in had no effect on basal vessel tone (0 mN above baseline, n=6), indicating that the contractions were due to the leukotriene and not to the solvent. The unresponsiveness of the nonatherosclerotic arteries was specific to the leukotrienes and not due to damage of the vessels, because both thromboxane A₂ and serotonin produced contractions in the same coronary arteries (data not shown).

Influence of Endothelium-Dependent Relaxing Factors on LTD₄ Responses

In the presence of indomethacin or in vessel segments in which the endothelium had been removed, nonatherosclerotic coronary arteries remained unresponsive to LTD₄ (0 mN, n=6). When nonatherosclerotic arteries were preconstricted with the thromboxane analogue U46619 or prostaglandin F₂α, LTD₄ (1 μmol/L to 0.1 μmol/L) failed to induce relaxations in the arteries (data not shown). The fact that there were no relaxation responses to LTD₄ was not because of damage to the endothelium, because the endothelium-dependent vasodilator substance P (10 nmol/L) induced relaxations in preconstricted coronary artery segments (data not shown). These results confirm our previous findings, which showed that LTC₄ responses in atherosclerotic coronary arteries were not influenced by the endothelium.²⁴

Low-Resolution Autoradiography of Atherosclerotic and Nonatherosclerotic Coronary Arteries

Qualitative low-resolution autoradiography images showed evidence of [³H]-LTC₄ (top) and [³H]-LTD₄ (bottom) binding to both atherosclerotic and nonatherosclerotic coronary arteries (Figure 2). In atherosclerotic vessels, tritiated LTC₄ appeared to show the greatest amount of binding at 1 nmol/L. In both nonatherosclerotic and atherosclerotic arteries, the degree of nonspecific binding (in the presence of excess unlabelled leukotriene) was high (50% to 80%), particularly at 3 nmol/L. To quantitatively assess the degree of specific binding to establish any differences in the amount of binding in the two vessel types (particularly at the low concentrations in which binding was too low to image), we estimated the amount of binding to both vessels by counting tritium levels. Counts of [³H]-LT showed a significant degree of specific [³H]-LTC₄ binding to atherosclerotic coronary artery (Figure 3, upper panel, 0.3 and 1.0 nmol/L; P<.05), with no evidence of concentration-dependent specific binding of [³H]-LTC₄ to nonatherosclerotic coronary artery (Figure 3, lower panel). Similarly, no significant concentration-dependent [³H]-LTD₄ binding was observed.

Figure 1. Leukotriene contractions in human atherosclerotic (solid symbols) and nonatherosclerotic (open symbols) epicardial coronary arteries. The curves show the contractions elicited by LTC₄ (open circles, n=8; solid circles, n=11) and LTD₄ (open squares, n=8; solid squares, n=11). The response is presented as a percentage of the response to 90 mmol/L KCl. All values are mean±SEM. (Eₘₐₓ values for LTC₄ versus LTD₄ [solid symbols], P<.05).

Figure 2. Representative low-resolution autoradiography images of tritiated LTC₄ and LTD₄ to human atherosclerotic (top) and nonatherosclerotic (bottom) epicardial coronary arteries. Total (TOT) [³H]-LTC₄ or [³H]-LTD₄ was achieved by incubating coronary artery segments in increasing (left to right) concentrations of radiolabeled leukotriene (0.3 to 3.0 nmol/L). Nonspecific binding (NSB) was obtained by incubating tissue in the presence of an excess of unlabelled leukotriene (1 μmol/L). Scale bar, 1 mm.
specific binding was evident in atherosclerotic (Figure 4, upper panel) or nonatherosclerotic vessels (Figure 4, lower panel).

High-Resolution Autoradiography of Atherosclerotic Coronary Arteries
Because LTC$_4$ was the only leukotriene that showed significant specific binding in atherosclerotic coronary arteries, we used high-resolution autoradiography to identify the cell types to which LTC$_4$ was binding. High-resolution images of atherosclerotic vessels showed dense [3H]-LTC$_4$ binding that was mainly localized to the medial smooth muscle cells, with less binding to adventitia (data not shown). In atherosclerotic coronary arteries, there was also additional binding to regions of intimal proliferation and very dense binding to areas of plaque (Figure 5). There was no evidence of [3H]-LTC$_4$ binding to the endothelium.

5-LO, FLAP, and LTA$_4$ Hydrolase Expression in Human Coronary Arteries
Immunocytochemical staining demonstrated macrophages in the adventitia of nonatherosclerotic vessel segments (Figure 6), with greater numbers in the atherosclerotic vessels (data not shown). In addition, some macrophages were also present in the media, whereas areas of intimal proliferation associated with the atherosclerotic coronary arteries were abundant with macrophages (Figure 7). Generally increased numbers were seen in the intima with increasing severity of disease. The more-advanced diseased arteries also had a few macrophages in the media. Cells positive for 5-LO, FLAP, and LTA$_4$ hydrolase were also seen in the adventitia and in areas of intimal proliferation that corresponded to the distribution of the macrophages (Figure 7). In these areas, 5-LO labeled the greatest number of cells, with FLAP present in a smaller percentage and LTA$_4$ hydrolase generally demonstrating the least number of positive cells. All negative control sections had no staining. Thus, compared with the nonatherosclerotic vessels (Figure 6), atherosclerotic arteries (Figure 7) contained a greater number of infiltrating macrophages and hence a greater amount of enzymatic machinery to produce leukotrienes. An unexpected finding was that the medial smooth muscle cells of both vessel types were also positive with all three leukotriene antibodies to a similar extent.
In preliminary experiments, we also found some positive staining for 5-LO, FLAP, and LTA4 hydrolase in the cultured smooth muscle cells derived from human coronary arteries (data not shown). The degree of positive staining was variable between smooth muscle cell cultures derived from different patients.

**Discussion**

These results show for the first time that the presence of atherosclerosis in human coronary arteries specifically augments contractions to cysteinyl leukotrienes and provides an enzymatic capacity within the vessel wall in the form of infiltrating macrophages and possibly smooth muscle cells to produce leukotrienes that could contribute to the hyperreactivity of atherosclerotic vessels. Hyperreactivity of human atherosclerotic coronary arteries to LTC4 and LTD4 was unaffected by endothelium-derived mediators. Previous reports have shown increased responsiveness of atherosclerotic arteries to serotonin that was unaffected by the endothelium. In those studies, hyperreactivity was reported to involve an increased responsiveness of the receptor or signal transduction system that was not apparent in the receptors.
present in the nondiseased arteries. Our present findings provide no evidence of cysteinyl leukotriene receptors in nonatherosclerotic epicardial coronary arteries, as suggested by the inability to contract to LTC4 or LTD4 and confirmed by the absence of a significant number of specific [3H]-leukotriene binding sites. In contrast, atherosclerotic vessels responded with potent contractions to LTC4 and with smaller contractions to LTD4, and a significant number of specific [3H]-LTC4 binding sites were present in the atherosclerotic vessels. These results confirm and extend our previous findings and suggest a novel mechanism whereby specific leukotriene receptors associated with atherosclerotic vessels may explain the augmented response to these leukotrienes.

There was considerable variation of specific [3H]-LTC4 and [3H]-LTD4 binding sites among the tissues, with only atherosclerotic coronary arteries exhibiting significant specific, concentration-dependent [3H]-LTC4 binding. Other studies have failed to detect any [3H]-LTD4 binding to dog aorta and bovine coronary artery, although [3H]-LTC4 bound at a relatively high level. Using [3H]-LTC4, several other studies have demonstrated the existence of a specific LTC4 binding site in membrane preparations of guinea pig, rat, and human lung. However, these data are difficult to interpret owing to a large number of independent LTC4 specific binding sites reported to be present in the membranes under investigation. The relevance of the LTC4 binding sites is still unclear, with results from many groups supporting the conclusions of an early report describing glutathione-S-transferase as the LTC4 binding protein. It is possible that a percentage of LTC4 and LTD4 binding in atherosclerotic and nonatherosclerotic vessels, particularly at 3 nmol/L (which showed the highest degree of nonspecific binding), may be attributable to nonspecific binding to nonreceptor proteins such as glutathione-S-transferase. Nonetheless, our functional and binding data suggest that LTC4- and LTD4-induced contractions of atherosclerotic coronary arteries occur via a leukotriene binding site specific for LTC4. Future functional and binding studies, using competition assays with different structural classes of leukotriene antagonists, should clarify whether these binding sites in atherosclerotic coronary arteries represent a distinct LTC4 receptor.

Examination of high-resolution autoradiographs of atherosclerotic coronary arteries revealed dense [3H]-LTC4 binding to the medial smooth muscle cells and regions of intimal proliferation and plaque formation. This is interesting in light of the recent work of Brezinski et al, who showed that angioplasty triggers intracoronary leukotriene production and who suggested that plaque rupture may be the stimulus triggering the appearance of these vasoactive compounds and that they may be derived from the atherosclerotic plaque itself or from the interaction of the released plaque debris with peripheral blood cells. There is now evidence that localized chronic inflammatory processes within the atherosclerotic plaque, rather than the endothelium, are responsible not only for plaque rupture itself but also for the hyperreactivity of these vessels to vasoconstrictor stimuli. The enhanced reactivity of the epicardial coronary arteries from IHD patients observed here, together with the evidence of leukotriene binding to plaque, appears to suggest that a local or systemic release of leukotrienes in response to tissue injury might contribute to spasm of a coronary vessel segment and/or precipitate a plaque rupture.

Human coronary arteries not only have the ability to contract to leukotrienes, they also have the capacity to produce leukotrienes. Previous work has shown that human and canine coronary arteries can produce leukotrienes when stimulated with calcium ionophore or treated with arachidonic acid. In the present study, we identified staining of 5-LO,
FLAP, and LTA₄ hydrolase that appeared to be associated with macrophages. The amount of staining for each leukotriene protein was increased in the atherosclerotic vessels and appeared to correlate with the presence of increased numbers of macrophages. Monocyte/macrophage recruitment to the vascular intima followed by foam cell transformation is a crucial early step in the development of atherosclerosis, and there is increased evidence that leukotrienes can play a role in this process. For example, 5-LO inhibitors can prevent the uptake of cholesterol esters into monocytes and macrophages in vitro. In addition, oxidized LDL can increase 5-LO activity in a mononuclear cell line, suggesting that in vivo oxidized LDL may play an important role in the upregulation of the 5-LO pathway. This is important because LDL is known to stimulate leukotriene production in monocytes. Furthermore, we have recent data to suggest that there is an overproduction of LTB₄ in patients with hypercholesterolemia (unpublished data from our laboratory, 1998). Leukotriene B₄ is chemoattractant for monocytes and can cause increased adhesion of leukocytes to the vascular endothelium. Taken together, the above evidence suggests that activation of the macrophage 5-LO pathway may play an important role in the inflammatory response associated with migration and transformation into foam cells of macrophages within the vessel intima. The importance of the 5-LO pathway in inflammation has recently been highlighted in 5-LO and FLAP knockout mice in studies that showed a blunted inflammatory response to topical arachidonic acid and platelet activating factor–induced shock compared with controls.

In conclusion, we present evidence of a novel mechanism in which atherosclerosis is associated with the appearance of a leukotriene receptor(s) capable of inducing hyperreactivity of human epicardial coronary arteries in response to LTC₄ and LTD₄. Because heart tissue has the capacity to both produce and respond to leukotrienes and because patients with coronary artery disease have raised levels of leukotrienes, the present findings suggest that endogenous leukotrienes may play an important role in the pathogenesis and clinical manifestations of atherosclerosis.

References


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