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Article

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The relationship of hydroxyeicosatetraenoic acids and F_2-isoprostanes to plaque instability in human carotid atherosclerosis

Ziad Mallat,[1] Tatsuji Nakamura,[2] Jeanny Ohan,[3] Guy Lesèche,[3] Alain Tedgui,[1] Jacques Maclouf,[4] and Robert C. Murphy[2]

[1]Institut National de la Santé et de la Recherche Médicale, Unité 141, IFR Circulation, Hôpital Lariboisière, 75475 Paris, France
[2]National Jewish Medical and Research Center, Department of Pediatrics, Division of Basic Sciences, Denver, Colorado 80206, USA
[3]Service de Chirurgie Thoracique et Vasculaire, Hôpital Beaujon, 92110 Clichy, France
[4]Institut National de la Santé et de la Recherche Médicale, Unité 348, IFR Circulation, Hôpital Lariboisière, 75475 Paris, France

Address correspondence to: Robert C. Murphy, National Jewish Medical and Research Center, 1400 Jackson Street, Denver, Colorado 80206, USA. Phone: (303) 398-1849; Fax: (303) 398-1694; E-mail: murphyr@njc.org

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Introduction

Atherosclerosis is a chronic disease of complex etiology ultimately resulting in arterial occlusion and acute thrombosis (1–3). There is a growing body of evidence suggesting that oxidant stress, in particular oxidative modification of low-density lipoproteins (LDLs), is involved in the initial events of atherogenesis (4–6). Lipids, including cholesterol esters and phospholipids, constitute 75%–80% of this lipoprotein, and formation of oxidized lipids derived from LDLs is thought to play a central role in mediating atherosclerotic vascular disease (5). Arachidonic acid and linoleic acid constitute the majority of the polyunsaturated fatty acids present within LDLs. These lipid substances are the most susceptible to oxidative modification, and they participate in free radical propagation reactions initiated during oxidant stress. The exact nature of the critically important oxidized components of LDLs, including lipids, has been the matter of considerable conjecture, with lines of evidence suggesting an important role for cholesterol ester hydroperoxides, including those derived from direct oxidation by 15-lipoxygenase, as well as oxidized products derived from glycerophospholipid components (7, 8).

Lipoxygenases are a family of closely related nonheme iron–containing dioxygenases that insert molecular oxygen into polyenoic fatty acids such as arachidonic acid or linoleic acid and convert them into hydroperoxide products (9). Lipoxygenases, and in particular 15-lipoxygenase, have been found expressed in human atherosclerotic arteries (10–12) and have been implicated in the oxidative modification of LDL. The action of 15-lipoxygenase on lipoproteins and membrane phospholipids may initiate a concerted interaction between enzymatic-derived lipid peroxidation and nonenzymatic lipid peroxidation. There are also suggestions that 15-lipoxygenase may play an antiatherogenic role (13).

Many investigations of LDL lipid peroxidation have focused on the production of malondialdehyde and related substances that represent terminal, stable products, but these low-molecular-weight products are by-products of the initial oxidation of a target lipid substance. More recently, a direct investigation of the formation of oxidized cholesterol esters (14, 15), as well as oxidized glycerophospholipids, has become possible because of advances made in the ability to structurally characterize such products while still present in complex lipid mixtures.

Table 1
Patient characteristics

	Asymptomatic Patients (n = 18)	Symptomatic Patients[A] (n = 12)
Age	73 ± 2	71 ± 3
Gender	Male (14)	Male (8)
Hypertension[B]	11	8
Hypercholesterolemia[C]	8	5
Diabetes	3	1
Currently smoking	6	3

[A]These patients presented with transient or persistent ischemic cerebral attack 8–45 days before endarterectomy.

[B]Number of subjects with clinical hypertension being treated with antihypertensive agents.

[C]Number of subjects with clinical hypercholesterolemia being treated with lipid-lowering drugs.

Initial nonenzymatic oxidation of esterified arachidonic acid in cellular membranes is known to yield a variety of stable products ranging from monohydroperoxyeicosatetraenoate (esterified hydroperoxyeicosatetraenoic acids [HPETEs]), epoxyeicosatetraenoate (esterified epoxyeicosatetraenoic acids [EETs]), and monohydroxyeicosatetraenoate (esterified hydroxyeicosatetraenoic acids [HETEs]) (16–18), to the considerably more complex F_2-isoprostanes (IPF_2), which are a family of free radical–derived prostaglandin $F_{2\alpha}$ isomers formed *in situ* within phospholipids (19–21). Such complex lipid products, as well as those derived from linoleate, have been suggested to be both sensitive and specific indexes of nonenzymatic lipid peroxidation taking place *in vivo* (22). Indeed, enhanced excretion of 8-iso-$PGF_{2\alpha}$ and other IPF_2 in urine has been associated to an index of lipid peroxidation in human vascular disease (20), and recently, Pratico *et al.* (23) localized F_2-isoprostanes in human carotid atherosclerotic plaques**.** Most investigations, however, have focused attention on the presence of a selected oxidized product derived from either LDL cholesterol esters or glycerophospholipids, and little is known concerning the relative quantity of oxidized polyunsaturated fatty acids, in particular those derived from arachidonic acid, present within atherosclerotic lesions.

Nonenzymatically formed oxidized arachidonic acid products including F_2-isoprostanes, HPETEs, and HETEs express a variety of biologic actions through specific receptors; these substances could be liberated from oxidized phospholipids after ester hydrolysis. This study was undertaken to examine the relative production of HETEs and F_2-isoprostanes within normal human arterial samples and in plaques removed from atherosclerotic lesions. Furthermore, the quantity of these products of arachidonic acid oxidation were examined in relationship to plaque instability, an important issue that had not been explored previously.

Methods

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$),8-epi-$PGF_{2\alpha}$, oxo-ETEs, HETEs, EETs, and [$^2H_4$]$PGF_{2\alpha}$ standards were purchased from Cayman Chemical (Ann Arbor, Michigan, USA). [^{3}H]arachidonic acid (40 mCi/mmol) was obtained from Du Pont NEN Research Products (Boston, Massachusetts, USA). The internal standard, [$^{18}O_2$]12-HETE, used for mass spectrometric quantitation, was prepared by the catalytic exchange of the carboxylic acid oxygen atoms with $H_2{}^{18}O$ using butyryl cholinesterase as described previously (24). All solvents used were of HPLC grade, and other reagents were of the highest grade commercially available.

Patient classification. Thirty patients were recruited to participate in the study and were classified according to clinical symptoms in two separate groups. The first group included 12 patients (8 men; 71 ± 3 years old) who presented with clinical symptoms of cerebral ischemic attack (symptomatic patients, group 1). Endarterectomy was performed 8–45 days after the onset of clinical symptoms in these patients. The second group included 18 patients (14 men; 73 ± 2 years old) who had an asymptomatic carotid stenosis (asymptomatic patients, group 2). Asymptomatic carotid stenosis was detected on the basis of systematic clinical examination of patients with coronary or peripheral disease, and its severity was determined using repeated Doppler echography by an experienced validated echographist. The asymptomatic patients never had an ischemic episode in the territory of the carotid stenosis, but carotid endarterectomy has been shown to be beneficial in these patients, as shown by Asymptomatic Carotid Atherosclerosis Study (ACAS) investigators (25–27). Percentage of carotid diameter reduction (60%–95%) and risk factors, including age, gender, diabetes, hypercholesterolemia, hypertension, and cigarette smoking did not differ between the two groups (Table 1). By the time of surgery, all patients were taking aspirin. Normal arterial tissues (three internal mamillary arteries and one uterine artery) were obtained in sizes similar to those used for the atherosclerotic plaques and served as controls. These control samples were devoid of plaques but were from coronary patients.

Biologic sample preparation. Surgically removed fragments of the carotid or control arteries ranged from 2 mm to 1 cm in length for a total wet weight of 100 mg to 1 g. They were snap-frozen at –70°C with liquid nitrogen and stored frozen before extraction in Paris. A double-blind code was used to identify each sample during the sample extraction and analysis. After all samples had been analyzed, each sample was identified according to group classification.

Lipid extraction and ester hydrolysis. Lipids were extracted in Paris following the method of Rose and Oklander (28). Briefly, isopropanol (11 ml) and butylated hydroxytoluene (0.2 mmol/l) were added to the plaques or arterial samples. In some experiments, the sample was homogenized with a polytron at this step. The mixture was then incubated for 1 h with frequent vortexing that was followed by the addition of chloroform (7 ml). After vortexing, the mixture was allowed to stand for 1 h; then it was centrifuged (500 *g*) for 30 min and the supernatant separated. The organic extracts were stored at –70°C for up to 2 months during the patient collection phase of the study and before shipping to Denver. This extraction

Table 2
Levels of total esterified HETEs and total F_2-isoprostanes in normal arteries and atherosclerotic plaques

	Normal arteries (n = 4)	Atherosclerotic plaques (n = 30)
Total HETEs (pmol/μmol lipid phosphorus)	97.0 ± 81	1303 ± 141
Total F_2-isoprostanes (pmol/μmol lipid phosphorus)	Not detected	55.2 ± 10.4

$P < 0.01$

protocol would extract phospholipids as well as the less abundant cholesterol esters containing esterified arachidonate.

In Denver, an aliquot of the extracted lipids (10%) was dried under vacuum and then hydrolyzed by the addition of isopropanol (2 ml) and 1 N sodium hydroxide (2 ml) at room temperature for 90 min. The samples were then acidified (pH 3.0) with 2 N hydrochloric acid, during which time [^{2}H]PGF$_{2\alpha}$ and [^{18}O]12-HETE (10 ng each) were added to each sample as internal standards. Free fatty acids were then extracted twice with hexane (5 ml). An identical aliquot was taken for a total phosphorus determination (29).

In separate experiments, normal mouse aortae samples (0.1–0.2 g; n = 3) were extracted and saponified, as described earlier, in the presence of [^{3}H]arachidonic acid (1 μCi) and butylated hydroxytoluene (BHT; 0.2 mM) to assess generation of oxidation products during sample preparation before mass spectrometric analysis. A further control study was carried out with aorta samples (n = 3) that were extracted after addition of [^{3}H]arachidonate (1 μCi), 0.2 mM BHT, and 100 ng 15-hydroperoxy eicosatetraenoic acid. No radiolabeled arachidonate oxidation products (specifically HETEs and HPETEs) as separated by TLC could be detected by radioactivity counting. This lack of arachidonate oxidation during extraction and work-up was consistent with control samples (human mamillary artery and uterine artery) that had low levels of esterified HETE and barely detectable quantities of esterified isoprostanes (see Results).

Mass spectrometry. Quantitation of HETEs, EETs, oxo-ETEs, and F$_2$-isoprostanes were performed using online reverse-phase HPLC tandem mass spectrometry (LC/MS/MS). Hexane extracts were taken to dryness under a stream of nitrogen and then reconstituted in 100 μl methanol. For the analysis of HETEs and EETs, water (50 μl) containing 0.05% acetic acid adjusted to pH 5.7 using ammonium hydroxide (mobile phase A) was added to 50 μl of the sample aliquot just before analysis. For the analysis of oxo-ETEs and F$_2$-isoprostanes, 100 μl of mobile phase A was added to 50 μl of sample derived from the sample just before analysis. For both analytical protocols, the diluted sample (50 μl) was analyzed by reverse-phase HPLC using a Prodigy C-18 column (1 × 250 mm, 5 μm ODS, 100; Phenomenex, Rancho Palos Verdes, California, USA) at a flow rate of 50 μl/min. The separation of HETEs and EETs was carried out using a linear gradient from 20% mobile phase A to 100% mobile phase B (acetonitrile/methanol, 65:35) over 15 min. The analysis of oxo-ETEs and F$_2$-isoprostanes was carried out in a separate run with a three-step linear gradient starting from 40% B to 50% B over 10 min, then to 90% B over 1 min, followed by 100% B over 4 min. In each analysis, the entirety of the HPLC column effluent was introduced into the electrospray mass spectrometer. Mass spectrometric analyses were performed on a Sciex API III$^+$ triple quadrupole mass spectrometer (PE-Sciex, Thornhill, Ontario, Canada) in the negative-ion mode using multiple-reaction monitoring (MRM) and monitoring the transitions m/z 319 → 115 for 5-HETE; m/z 319 → 151 for 9-HETE; m/z 319 → 155 for 8-HETE and 8,9-EET; m/z 319 → 167 for 11-HETE; m/z 319 → 191 for 5,6-EET; m/z 319 → 208 for 12-HETE and 11,12-EET; m/z 319 → 219 for 15-HETE and 14,15-EET; m/z 323 → 183 for [^{18}O$_2$]12-HETE; m/z 317 → 113 for 15-oxo-ETE; m/z 317 → 153 for 12-oxo-ETE; m/z 317 → 203 for 5-oxo-ETE; m/z 353 → 309 for F$_2$-isoprostanes; and m/z 357 → 313 for [^{2}H$_4$]PGF$_{2\alpha}$.

A dwell time of 425 ms was used for each ion transition, for a total scan time of 3.0 s for a selection of eight mass transitions in each MRM protocol. Argon was used in the collision cell at a collision gas thickness to 240 × 10^{13} molecules/cm^2. The spray voltages used were 3,000 or 3,400 V for HETEs/EETs or oxo-ETEs/ F$_2$-isoprostanes, respectively. The orifice potential was set at –55 V, with an offset voltage of 11 or 20 eV for HETEs/EETs or oxo-ETEs/F$_2$-isoprostanes, respectively. Nitrogen was used as the curtain gas in the electrospray interface, and zero air was used as the nebulizer gas to suppress a glow discharge from the electrospray needle.

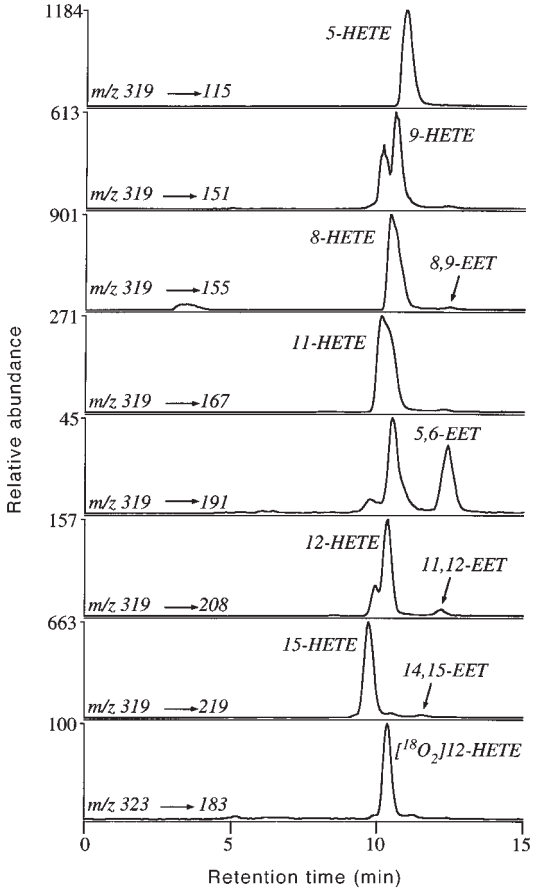

Figure 1
LC/MS/MS analysis of six HETEs and four EETs isolated from surgically removed atherosclerotic plaques. Lipids extracted from the plaques were hydrolyzed with 1 N NaOH, acidified, and then analyzed by online reverse-phase HPLC with MRM of the specific ion transitions indicated at each ion profile trace. The signal intensity for each MRM trace is indicated for each ion transition and normalized to the signal obtained for internal standard [^{18}O$_2$]12-HETE added (10 ng) to each lipid extract. The elution of each eicosanoid from the HPLC column is indicated. *EETs*, epoxyeicosatetraenoic acids; *HETEs*, hydroxyeicosatetraenoic acids; *LC/MS/MS,* reverse-phase HPLC tandem mass spectrometry; *MRM,* multiple-reaction monitoring.

Quantitative analysis of the target eicosanoids was carried out using stable isotope dilution using either [^{18}O$_2$]12-HETE as the internal standard for the EETs, oxo-ETEs, and mono-HETEs, or [^{2}H$_4$]PGF$_{2\alpha}$ for F$_2$-isoprostanes. Standard curves were generated ratioing the abundance of the ion transitions from each target eicosanoid to that of the internal standard as a function of quantity of analyte. These curves were found to be linear over the range of 0.1–100 ng injected on column. The LC/MS/MS quantitative assay for isoprostanes was based on determining the ratio of the abundance of the ion transition (m/z 357 → 313) for the deuterated PGF$_{2\alpha}$ internal standard divided into the area under the curve for the corresponding transition (m/z 353 → 309) observed for the family of isoprostanes. For the isoprostanes, multiple HPLC peaks were readily observed, owing to the presence of multiple isoprostane family members.

Table 3
Levels of total esterified HETEs and total F_2-isoprostanes in human subjects with asymptomatic and symptomatic plaques

	Asymptomatic plaques (n = 18)	Symptomatic plaques (n = 12)	
Total HETEs (pmol/μmol lipid phosphorus)	1,002 ± 107	1,738 ± 274	P < 0.01
Total F_2-isoprostanes (pmol/μmol lipid phosphorus)	50.1 ± 10.9	62.8 ± 20.8	N.S.[A]

[A]Not Significant

Statistical analysis. Results are given as mean ± SEM. Comparisons between normal and atherosclerotic plaques and between stable plaques (asymptomatic patients) and unstable plaques (symptomatic patients) were done using Student's t test. P < 0.05 was considered statistically significant.

Results

A representative LC/MS/MS and MRM analysis for HETEs and EETs is shown in Fig. 1. All samples from atherosclerotic plaques showed the presence of multiple HETE isomers, including 5-, 9-, 8-, 11-, 12-, and 15-HETE, liberated from the extracted lipids by the saponification step. For some samples, a detectable quantity of an epoxyeicosatetraenoic acid was observed, and in the sample shown (Fig. 1), 5,6-EET was particularly prevalent. Normally EETs represented a small component of the oxidized fatty acyl groups derived from arachidonic acid in the atherosclerotic plaque extracts. Because the calibration curves for this MRM assay for all compounds (including HETEs and EETs) showed an excellent correlation in the range of 0.1–100 ng with synthetic standards, these ion-monitoring profiles could be used along with the elution of the $[^{18}O_2]$12-HETE added as an internal standard, for a quantitative description of the formation of these components.

LC/MS/MS analysis of isoprostanes derived from a representative atherosclerotic plaque is shown in Fig. 2. The elution of 8-iso-$PGF_{2\alpha}$ and $PGF_{2\alpha}$ was found to occur at 11.3 and 13.4 minutes, respectively, from reverse-phase HPLC column (data not shown). Because these two prostanoids represent only two of the potential 64 regioisomers constituting F_2-isoprostanes, the ion profile between 10.0 and 15.2 minutes was integrated and taken for the quantitative measure of the total isoprostane components in the extract. The oxo-ETEs eluted between 17.5 and 19 minutes under these reverse-phase HPLC conditions based on the analysis of synthetic standards. In none of the plaque samples were oxo-ETE components observed at the detection limit of this assay, 0.03 ng injected on the column.

Elevated levels of HETEs and isoprostanes in atherosclerotic tissues. High levels of isomeric HETEs were detected in all atherosclerotic plaques (1303 ± 141 pmol/μmol lipid phosphorous) (Table 2). The most abundant monooxygenated eicosanoid was found to be 9-HETE, which accounted for 21.4 ± 1.9% of the total HETEs. The least abundant HETE was 15-HETE, which accounted for 11.1 ± 1.0% of total HETEs). In contrast to the atherosclerotic tissue, very low levels of HETEs were detected in normal human artery extracts (97 ± 81 pmol/μmol lipid phosphorous; P < 0.01 in comparison with atherosclerotic plaques; Table 2).

Isoprostanes were barely detectable in normal arteries (Table 2). However, these compounds were present in significant amounts in atherosclerotic plaques (55 ± 10 pmol/μmol lipid phosphorous) (Table 2).

Elevated levels of HETEs in symptomatic patients. Levels of total HETEs were significantly elevated in plaques retrieved from symptomatic patients (group 1) compared with those retrieved from asymptomatic patients (group 2) (1,738 ± 274 vs. 1,002 ± 107 pmol/μmol lipid phosphorous, respectively; P < 0.01; Table 3). Taken separately, the total amount of each HETE product was significantly increased in group 1 compared with the corresponding HETE product in group 2 (Fig. 3). The results were virtually unchanged when values were expressed relative to tissue weight (milligrams wet weight).

Levels of isoprostanes were elevated in group 1 compared with group 2, but the increase did not reach statistical significance whether expressed in milligrams of wet tissue or in micromoles of lipid phosphorus (62.8 ± 20.8 vs. 50.1 ± 10.9 pmol/μmol lipid phosphorus (Table 3). There was no significant difference between symptomatic and asymptomatic patients in the four EET regioisomers (33.6 ± 6.8 vs. 52.4 ± 18.9 pmol/μmol lipid phosphorus, respectively; n = 16, P = 0.75) or in oxo-ETEs levels (9.4 ± 1.4 vs. 10.9 ± 3.3 pmol/μmol lipid phosphorus, respectively; n = 30, P = 0.98).

Discussion

The major findings of this study are that (*a*) human atherosclerotic plaques contained substantially higher quantities of esterified HETEs compared with nonath-

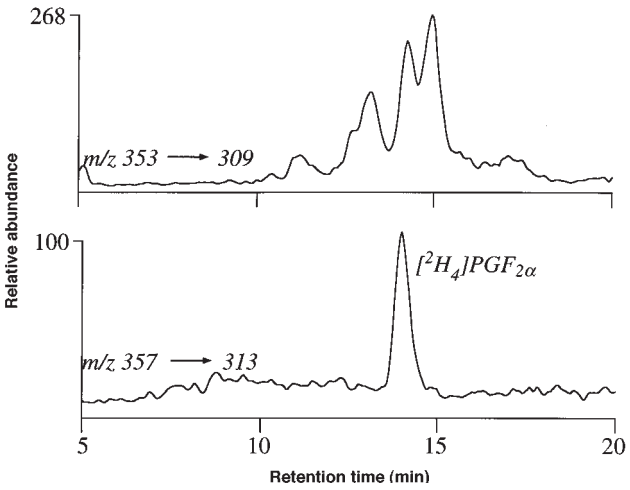

Figure 2
LC/MS/MS analysis for the loss of 44 u from the carboxylate anion of F_2-isoprostanes (*m/z 353 → 309*). Lipids extracted from atherosclerotic plaques were hydrolyzed with 1 N NaOH, acidified, and then analyzed by MRM using reverse-phase HPLC (see Fig. 1 legend). $[^2H_4]PGF_{2\alpha}$ (10 ng) was added as an internal standard, and the signal intensity for the ion transitions was normalized to the internal standard.

erosclerotic arteries; (*b*) plaque levels of esterified HETEs were much higher than those for EETs, oxo-ETE, and F_2-isoprostanes; and (*c*) most importantly, the absolute quantity of esterified HETEs in plaques derived from symptomatic patients was almost 1.7-fold higher than that derived from asymptomatic patients.

Previous studies have suggested that oxidant injury and lipids derived from oxidatively damaged lipoproteins may be critical components of human atherosclerosis. Recently, F_2-isoprostanes have been identified in extracts of human atherosclerotic plaque (23). Our studies confirm and extend these observations by demonstrating that atherosclerotic plaques also contain significant quantities of mono-HETEs esterified to lipids and small, but detectable, quantities of EETs. It should be noted, however, that in our studies, levels of total F_2-isoprostanes in plaques were 24 times lower than the HETEs in the same extracts. This finding suggests that F_2-isoprostanes are not the only potential markers for oxidative events taking place within atherosclerotic-derived lipids and that HETEs might be a more sensitive quantitative index of the oxidant injury. A similar observation has also been supported for the hydroxyoctadecadienoate products in atherosclerotic plaque samples derived from linoleate (22, 30).

Because morbidity and mortality of atherosclerosis are related to clinical manifestation of unstable plaques, we examined whether quantitative or qualitative modification of oxidized arachidonate derived from esterified lipids could be associated with changes in plaque development and stability. It has been suggested previously (28, 31, 32) that one F_2-isoprostane, namely 8-iso-$PGF_{2\alpha}$, is a mitogen and vasoconstrictor, suggesting that it may play a significant role in plaque evolution. The quantity of this single F_2-isoprostane isomer is similar to the level of EET esters but substantially less than that of total F_2-isoprostanes, and it is well below the levels of the HETE isomers observed in these atherosclerotic plaques. Moreover, HETEs, and in particular 5-HETE, are known to possess significant biologic activity, likely working through a specific G protein–linked receptor (33). Although 5-HETE is not the predominant HETE, it is much more abundant than all F_2-isoprostanes including 8-iso-$PGF_{2\alpha}$. Recent studies (34) have also suggested a unique and relevant activity for specific EETs in vascular responses. Therefore, the relevance of compounds other than 8-iso-$PGF_{2\alpha}$ as markers of oxidative damage and as products capable of potential biologic contributions *in vivo* should not be overlooked.

In our studies, levels of F_2-isoprostanes were not significantly increased in symptomatic patients compared with asymptomatic patients. However, levels of HETEs, which represent the major series of oxidized esters of arachidonic acid in our samples, were significantly elevated in those extracts originating from symptomatic patients. Clinical symptoms of cerebral and myocardial ischemia in patients with carotid and coronary atherosclerosis are predominant manifestations of unstable ruptured plaques (3, 35). Therefore, our results suggest that HETE-containing lipids may represent biologic markers that can be associated with plaque instability, thus linking the extent of oxidation (reflected by a quantitative index) to clinical symptoms. The failure to discriminate between the two populations in terms of F_2-isoprostanes could be partly attributed to the low number of individuals investigated in this study and to variations in the quantity of F_2-isoprostanes. No intergroup differences could be found for EETs and oxo-ETEs.

It should be noted that the quantity of HETE-containing lipids are expressed relative to total lipid phosphorus in the extracts and are not normalized to phospholipid arachidonate content. A previous investigation (36) found the quantity of arachidonate in cholesterol esters isolated from normal intima to be twofold lower than that found in the cholesterol esters isolated from plaque lesions, but the quantity of cholesterol ester arachidonate in fibrous plaque was found to be identical to that in gruel plaques. This previous investigation suggests that total arachidonate accumulation in the atherosclerotic plaques from both symptomatic and asymptomatic subjects may be responsible for some of the observed differences in HETE-containing lipids relative to control subjects, but it does not explain the observed difference between symptomatic and asymptomatic HETE-containing lipid content.

We did not observe any significant variation in the eicosanoid values related to the weight of the excised vascular tissue samples nor any variation in the quantity of eicosanoids when they were extracted by the organic solvent after prior homogenization of the tissues, suggesting that the oxidized phospholipids within the plaques were readily accessible by solvent extraction. Moreover, expression of the results either on a wet weight or total phosphorus basis did not reveal striking differences. Finally, given that the samples were extracted and analyzed blindly, the fact that there was a substantial difference between normal and pathological tissues and also between subgroups of pathological samples, constitutes additional support that any unavoidable contribution of sample oxidation during analytical procedures was minimal.

A particular emphasis has been placed on the potential pathophysiological role of the 15-lipoxygenase in atherosclerosis (11–13, 37). Contrary to other fatty acid oxyge-

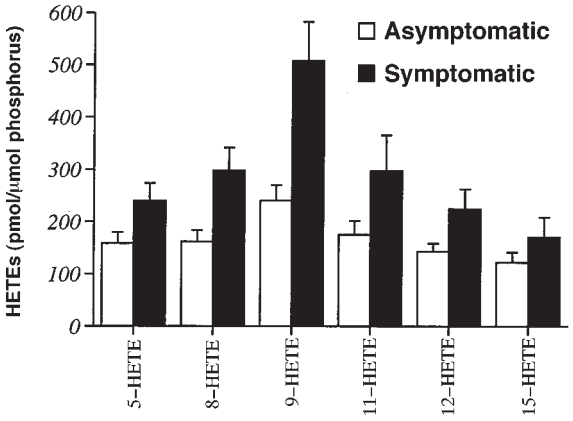

Figure 3
Distribution of HETE compounds in plaques retrieved from asymptomatic (18 stable plaques) and symptomatic (12 unstable plaques) patients. Each HETE, with concentration expressed on a per-mole-of-lipid-phosphorus basis, taken separately was significantly more abundant in unstable, compared with stable, plaques ($P < 0.01$).

nases, this enzyme is capable of oxidizing arachidonate or linoleate while it is still esterified in its glycerophospholipid substrate, thus not requiring prior hydrolysis of polyunsaturated fatty acids by phospholipases. Linoleic acid is the most abundant polyunsaturated fatty acid within LDLs (40%–45% total fatty acid), compared with only 5%–10% arachidonate. It is the preferred substrate of 15-lipoxygenase, leading to the formation of 13-hydroxyoctadecadienoic acid that has recently been reported (11, 31) in plaque samples similar to those used in the present work. In these studies, significant quantities of 5-, 12-, 15-HETEs were not reported, most likely due to a difference in methodology (HPLC compared with the more specific and sensitive LC/MS/MS here). We have identified a significant quantity of HETEs, including 15-HETE, in the plaques analyzed in this study.

Although we could not determine the stereochemistry of any of the HETEs because of their low absolute quantities available, it is noteworthy that the major oxygenated compound, 9-HETE, is not derived from a currently known enzymatic reaction. Because 9-HETE represented the major product of lipid peroxidation in this series of atherosclerotic samples and this HETE isomer was significantly associated with clinical symptoms, it is likely that nonenzymatic lipid peroxidation predominates in atherosclerosis. Such HETEs may constitute valuable markers of atherosclerosis progression from the stable to the unstable state, and it would be interesting to examine the effects of antioxidant therapies on plaque levels of HETEs. Although our results do not exclude the participation of 15-lipoxygenase in pathological processes linked to the genesis and/or progression of plaque, they support the importance of free radical processes in the formation of lipid peroxidation products. Nonenzymatic peroxidation of arachidonate leads to the formation of a family of six monohydroperoxy radical regioisomers as a racemic mixture. The radical can subsequently propagate radical reaction and be reduced to a family of hydroxyeicosatetraenoate esters, such as those reported here, after reduction via several enzymatic and nonenzymatic reactions. Furthermore, some reports in animals point to a protective role of 15-lipoxygenase against atherosclerosis development (13), and a recent study in humans (12) suggested that 15-lipoxygenase may be functionally silent in advanced human plaques and that specific enzymatic products may decompose or become a part of the distribution of large amounts of nonenzymatic lipid peroxidation products.

In conclusion, products of lipid peroxidation, and in particular those derived from arachidonic acid, have been found to be present in substantial amounts in human atherosclerotic plaques. HETEs were found to be major components and likely result from nonenzymatic lipid peroxidation of arachidonic acid within the plaque. The presence of HETEs appears to be related to the instability of the atherosclerotic plaque. Although some caution must be exercised in interpreting the results because the number of subjects studied was small, nevertheless, there was a remarkable difference in HETE-containing lipids between symptomatic, asymptomatic, and control samples. Control of HETE production, perhaps using antioxidants, may prove useful to ensure plaque stability.

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