Published in final edited form as:

Free Radic Biol Med. 2006 December 1; 41(11): 1678–1683.

Systemic elevations of free radical oxidation products of arachidonic acid are associated with angiographic evidence of coronary artery disease

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Abstract

Oxidant stress is widely believed to participate in cardiovascular disease pathogenesis. However, progress in defining appropriate systemic anti-oxidant targeted therapies has been hindered by uncertainty in defining clinically relevant systemic oxidant stress measures. In a case control study, 50 subjects with CAD (> 50% stenosis in one or more major coronary vessels) and 54 without CAD (< 30% stenosis in all major coronary vessels) were tested. Plasma was isolated and stored under conditions designed to prevent artificial lipid peroxidation. Systemic levels of multiple (n=9) specific fatty acid oxidation products including individual hydroxy-octadecadienoic acids (HODEs), hydroxy-eicosatetraenoic acids (HETEs), and F₂-Isoprostanes, were simultaneously measured by high performance liquid chromatography (HPLC) with on-line tandem mass spectrometry, along with traditional risk factors and C-reactive protein (CRP) levels. Of the markers monitored, only 9-HETE and F₂-Isoprostanes, both products of free radical-mediated arachidonic acid oxidation, were significantly elevated in patients with angiographically defined CAD (9-HETE, 8.7 ± 4 vs. 6.8 ± 4 $\mu\text{mol/mol}$ arachidonate, $p = 0.011$; and F₂-Isoprostanes, 9.4 ± 5 vs. 6.2 ± 3 $\mu\text{mol/mol}$ arachidonate, $p < 0.001$). In multivariable analyses with simultaneous adjustment for Framingham Risk Score and C-reactive protein, 9-HETE (4th quartile OR=4.8, 95% CI=1.3 to 17.1; $P = 0.016$) and F₂-Isoprostanes (4th quartile OR=9.7, 95% CI=2.56 to 36.9; $P < 0.001$) remained strong and independent predictors of CAD risk. Systemic levels of 9-HETE and F₂-Isoprostanes are independently associated with angiographic evidence of CAD and appear superior to other specific oxidation products of arachidonic and linoleic acids as predictors of the presence of angiographically evident coronary artery disease.

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Supported by National Institutes of Health grants P01 HL076491, P01 HL77107, HL70621, the American Heart Association and the Cleveland Clinic Foundation General Clinical Research Center (M01 RR018390). Dr. Shishehbor is supported in part by the National Institutes of Health, National Institute of Child Health and Human Development, Multidisciplinary Clinical Research Career Development Programs Grant K12 HD049091 and the National Institute of Health Loan Repayment Program.

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Keywords

Coronary artery disease; Oxidation; Oxidation products; F₂-Isoprostanes

INTRODUCTION

Oxidative reactions play a significant role in many biological conditions including chronic diseases such as atherosclerosis [1–7]. Oxidative modification of low-density lipoprotein and subsequent formation of foam cells are thought to be an initial step in atherogenesis. Multiple animal and in vitro studies have further supported the role of oxidative processes in virtually all phases of coronary artery disease (CAD), from foam cell formation to plaque rupture and thrombosis [1–9]. However, recent clinical data from large randomized studies have on the whole failed to demonstrate a significant reduction in clinical events after treatment with “antioxidant” interventions, such as supplementation with α -tocopherol [10].

A major concern with the design of most anti-oxidant intervention studies has been the lack of concomitant assessments of systemic measures of oxidant stress [8,11–14]. A major reason for this omission has been the lack of validated oxidant stress measures that are associated with cardiovascular risks and modulated by cardiovascular risk reducing therapies. Consequently, anti-oxidant supplementation studies have yet to utilize oxidant stress measures as enrollment criterion to define populations with proven heightened levels of oxidant stress. Similarly, randomized α -tocopherol supplementation trials have thus far failed to concomitantly demonstrate the magnitude of systemic anti-oxidant effect promoted in subjects included in the antioxidant arms of intervention trials, and their correlations with cardiovascular outcomes.

A large number of lipid and protein oxidation products have been described that might potentially serve as markers with which to assess oxidative burden and cardiovascular risks in vivo [8,11–14]. For example, we have recently shown that protein end products of specific oxidative pathways, such as those promoted by myeloperoxidase and nitric oxide (NO)-derived oxidants, are independently associated with CAD and modulated by 3-hydroxy-3-methylglutaryl CoA Reductase inhibitor (statin) therapy [15,16]. Initiation of lipid peroxidation and formation of an array of bioactive fatty acid oxidation products are widely held as critical steps in the atherosclerotic process. As far as we are aware, studies aimed at identifying structurally defined lipid peroxidation products within the systemic circulation that most strongly correlate with cardiovascular disease risks have not yet been reported. Herein we evaluated the relationship between systemic levels of multiple structurally distinct lipid peroxidation products of arachidonic and linoleic acids and angiographically defined CAD in order to identify those species of greatest potential clinical utility.

MATERIALS AND METHODS

Study Protocol

The population consisted of a consecutive sample of patients who underwent diagnostic coronary angiography at the Cleveland Clinic Foundation and met the definition of case and control as outlined below. Consenting subjects were enrolled as part of a clinical study named GeneBank, which is solely designed for the establishment of a clinical and tissue repository for research purposes [17]. Case patients were defined as those with stenoses of 50% or greater in one or more major coronary vessels demonstrated by coronary angiography. Control patients were those with no evidence of coronary artery disease or less than 30% stenoses on coronary angiography. All patients gave written, informed consent, and the Institutional Review Board of Cleveland Clinic Foundation approved the study protocol.

Blood Samples

Blood samples were collected into ethylenediaminetetraacetic acid (EDTA) tubes from fasting patients. Blood was immediately placed on ice or within a refrigerator, and samples centrifuged at 3500 rpm for 10 minutes at 4°C within 2 hr of collection. Plasma was then immediately stored under conditions to minimize artificial oxidation (i.e. with antioxidant cocktail, under inert atmosphere). Briefly, plasma was aliquoted into tubes containing butylated hydroxytoluene (100M final) and EDTA (100M final), overlaid with argon, snap frozen in liquid nitrogen, and stored at -80°C until analysis. Standard methods were used to measure fasting lipid profiles, and high-sensitivity C-reactive protein (CRP) was quantified by nephelometry (Dade Behring).

Mass spectrometry of individual oxidized lipids

High performance liquid chromatography (HPLC) with on-line electrospray ionization tandem mass spectrometry (LC/ESI/MS/MS) was employed to quantify total levels (free plus esterified) of multiple distinct oxidation products of arachidonic acid (AA) and linoleic acid, including individual hydroxy-eicosatetraenoic acids (HETEs), F₂-isoprostanes and hydroxy-octadecadienoic acids (HODEs), using established methods [18]. Briefly, 10 ng each of two deuterated internal standards, 12(S)-hydroxy-5,8,10,14-eicosatetraenoic-5,6,8,9,11,12,14,15-d₈ acid (12-HETE-d₈) and prostaglandin F_{2α} (PGF_{2α}-d₄) (Cayman Chemical Company, Ann Arbor, MI) were added to plasma samples, fatty acids were released by base hydrolysis with 0.2 M NaOH at 60°C for 30 min under argon atmosphere, samples acidified to pH 3.0 with 0.5 M HCl, and then fatty acids were extracted twice with 4 ml hexane. The combined hexane extracts were dried under N₂ flow, resuspended in 100 l methanol. One volume of H₂O was added to 20 volumes of the methanol-suspended sample, and then passed through a 0.22 μm filter (Millipore Corporation, Bedford, MA). Sample (50 l) was injected onto a C-18 column (2 × 250 mm, 5 μm ODS, 100Å; Phenomenex, Rancho Palos Verdes, CA) at a flow rate of 0.2 ml/min. Separations were performed using a gradient starting from 85% methanol over 15 min, then to 100% methanol over 1 min, following by 100% methanol for 15 min. HPLC column effluent was split so that only 50% was introduced into a triple quadrupole mass spectrometer (API 365, Applied Biosystems, Foster City, CA) with Ionics EP 10+ upgrade (Concord, Ontario, CA) interfaced to a Cohesive Technologies Aria LX Series HPLC multiplexing system (Franklin, MA).

Analyses were performed using electrospray ionization in negative-ion mode with multiple reaction monitoring of parent and characteristic daughter ions specific for each isomer monitored. The transitions monitored were mass-to-charge ratio (m/z): m/z 295 → 171 for 9-HODE; m/z 295 → 195 for 13-HODE; m/z 279 → 261 for LA; m/z 319 → 115 for 5-HETE; m/z 319 → 155 for 8-HETE; m/z 319 → 151 for 9-HETE; m/z 319 → 167 for 11-HETE; m/z 319 → 179 for 12-HETE; m/z 319 → 175 for 15-HETE; m/z 303 → 259 for AA; m/z 327 → 184 for 12-HETE-d₈; m/z 353 → 309 for F₂-isoprostanes; and m/z 357 → 313 for PGF_{2α}-d₄. Collision-induced dissociation was obtained using argon gas. The internal standard 12-HETE-d₈ was used to for quantification of HETEs, as well as to calculate extraction efficiencies of HODEs and HETEs (which control studies revealed were > 85%). The internal standard PGF_{2α}-d₄ was used to for quantification of F₂-isoprostanes.

Statistical Analysis

Data are presented as mean ± standard deviation for continuous measures, counts and percentages for categorical variables. Differences in data were compared using analysis of variance and Wilcoxon Rank-Sum tests for continuous measures, and chi-square and Fisher's exact tests for categorical variables. Logistic regression methods were performed and odds-ratios with 95% confidence intervals are presented. To facilitate examination of the association of lipid oxidation products with the presence of CAD, levels of each oxidation product within

the population were divided into quartiles. The associations between individual lipid oxidation products and angiographic evidence of CAD were then assessed in both unadjusted and adjusted models. In the multivariable model individual lipid peroxidation products were adjusted for the Framingham Risk Score and CRP. In addition, bootstrapping methods were utilized to validate the multivariate model. Spearman-rank correlations were used to examine associations between individual lipid oxidation products and cardiovascular risk factors. All analyses were performed using the SAS 8 System (Cary, NC).

RESULTS

Patient Demographics

The clinical and laboratory characteristics of study participants are shown in Table 1. Patients with CAD were older and had lower fasting total cholesterol and high density lipoprotein cholesterol levels. Furthermore, they had higher levels of CRP, and were more likely to use lipid lowering and other cardiovascular medications. These findings likely reflect confounding by indication bias, whereby patients with a higher level of disease are more likely to be taking medications.

Lipid peroxidation levels and prevalence of angiographically defined CAD

Of the lipid peroxidation products of arachidonic and linoleic acid monitored, plasma levels of total 9-HETE (8.7 ± 3.9 vs. 6.8 ± 3.6 , $p = 0.011$) and F₂-Isoprostanes (9.4 ± 4.6 vs. 6.2 ± 3.4 , $p < 0.001$) demonstrated the greatest significant increases in patients with CAD (Table 2). Rates of angiographic CAD were associated with increasing levels of 9-HETE quartiles (Q1=32%, Q2=50%, Q3=63%, Q4=62%, $p = 0.098$), and F₂-Isoprostanes quartiles (Q1=27%, Q2=46%, Q3=58%, Q4=77%, $p = 0.003$) (Figure 1). Patients in the highest quartile of either 9-HETE (unadjusted OR = 3.40; 95% CI: 1.07–10.8; $p = 0.038$) or F₂-Isoprostane (unadjusted OR = 9.05; 95% CI: 2.57–31.8; $p = 0.038$) had significantly increased risk of CAD compared to the lowest quartile (Figure 2). Patients in the highest quartile of CRP levels had a trend toward increased prevalence of angiographic CAD compared to the lowest quartile (OR=2.58; 95% CI: 0.84–7.91; $p=0.098$), with rates of CAD trending to increase with increasing CRP quartiles (Q1=40%, Q2=42.3%, Q3=57.7%, Q4=65.4%, $p=0.20$). Remarkably, none of the other structurally distinct lipid peroxidation products monitored were associated with the prevalence of angiographic CAD (Table 2).

Relationship between individual lipid peroxidation products and risks for angiographically defined CAD

Both F₂-Isoprostane and 9-H(P)ETE each demonstrated significant association with angiographic CAD, however, neither lipid peroxidation product demonstrated statistically significant correlations with traditional cardiovascular risk factors including low density lipoprotein cholesterol, high density lipoprotein cholesterol, or total cholesterol. Levels of both 9-H(P)ETE and F₂-Isoprostane remained significant predictors of CAD status following single-factor adjustments for individual traditional CAD risk factors (age, gender, history of diabetes, current smoking, history of hypertension, low density lipoprotein cholesterol, high density lipoprotein cholesterol, triglycerides) and log CRP (Table 3). In multivariable analyses with simultaneous adjustment for Framingham Risk Score and log CRP, both 9-HETE and F₂-Isoprostane remained strong and independent predictors of angiographic CAD risk (4th quartile OR=4.8, 95% CI: 1.3 to 17.1; $P = 0.016$ for 9-HETE; and 4th quartile OR=9.7, 95% CI=2.56 to 36.9; $P = 0.001$ for F₂-Isoprostanes) (Table 3). In addition, in multivariate models after adjusting for Framingham Risk Score plus F₂-Isoprostane, log CRP also remained significantly associated with CAD prevalence (OR=1.58, 95% CI=1.05–2.39; $P = 0.028$) (Table 3).

To confirm that F₂-Isoprostane and 9-HETE levels added to the predictive value of established markers of cardiovascular risk, we performed likelihood ratio tests on multivariate logistic regression models with and without these markers. Introducing F₂-Isoprostane, 9-HETE or CRP to a multivariate prediction model that included the Framingham Risk Score significantly improved the prediction of angiographic CAD in the cohort (Table 3).

DISCUSSION

Oxidative stress and lipid peroxidation are widely believed to play a significant role in the atherosclerotic process [2,9,19,20]. And yet, prospective randomized intervention studies utilizing anti-oxidant supplementation such as α -tocopherol have, on the whole, failed to demonstrate clinical benefit [8,10]. Importantly, in human studies where large doses of α -tocopherol have been used and oxidative products monitored, no reduction in systemic markers of oxidant stress have been noted [21]. Numerous potential reasons for lack of benefit among randomized antioxidant studies have been suggested [8,11]. For example, α -tocopherol and vitamin C serve as reversible redox-active agents that can both promote and inhibit oxidation reactions, depending upon the situation [22,23]. Further, a growing body of evidence suggests that many of oxidation processes that are operational within human atheroma, such as those mediated by nitric oxide-derived oxidants and myeloperoxidase [22,24,25], are not readily inhibited by α -tocopherol [26,27].

Progress in the anti-oxidant supplementation field has been hindered by both a lack of consensus on what the optimal systemic indices of oxidant stress to monitor are, as well as methodological obstacles (in terms of how to measure oxidation products, or prevent their artificial generation) [11–13,28–31]. There is a growing consensus within the oxidation field that one should first identify individuals with increased systemic indices of oxidant stress before the appropriate anti-oxidant interventions may be evaluated. Clinical efficacy of a presumed antioxidant cannot be evaluated without concomitant evidence of anti-oxidant effect following supplementation in subjects at presumed higher risk because of elevated indices of oxidant stress.

Herein we examined the relationship between multiple distinct products of lipid peroxidation by free radical mediated processes and the correlation with angiographic evidence of CAD. The arachidonic acid oxidation products 9-HETE and F₂-Isoprostane appeared superior to alternative free radical generated arachidonic acid and linoleic acid oxidation products as potential indices of CAD risk as defined by coronary angiography. Remarkably, both of these free radical generated lipid peroxidation products demonstrated strong and independent correlations with angiographic evidence of CAD, even after adjustments for Framingham Risk Score and CRP. Hence, 9-HETE and F₂-Isoprostane appear to serve as independent lipid oxidation markers that provide additive value that goes beyond established and novel risk factors for predicting angiographic evidence of CAD.

While F₂-Isoprostane, free radical-catalyzed products of arachidonic acid, have been widely studied as potential biomarkers of oxidant stress [2,14,31–33], there are no investigations, as far as we are aware, that have simultaneously monitored multiple distinct lipid oxidation products from blood to determine which are more tightly correlated with cardiovascular disease prevalence, and thus potentially of greatest clinical relevance. Remarkably, studies examining lipid oxidation species within excised human atheroma from symptomatic versus non-symptomatic carotid endarterectomy specimens identified 9-HETE, and less so F₂-Isoprostane, as abundant fatty acid oxidation products within atheroma from symptomatic vascular lesions [34]. Other studies have documented the presence of F₂-Isoprostane as lipid oxidation products enriched within atheroma [32]. Whether plasma levels of F₂-Isoprostane, 9-HETE, or alternative free radical oxidation products of arachidonic acid or linoleic acid are independently

correlated with angiographically defined coronary artery disease had not been systematically examined. In our study, of the multiple free radical lipid oxidation products monitored, only 9-HETE and F₂-Isoprostane levels were significantly associated with the presence of CAD, even after adjustment for Framingham risk score and CRP. Remarkably, this association was observed despite multiple corrections for known cardiac risk factors, since prior studies demonstrate levels of F₂-Isoprostane are elevated in conditions like smoking and hyperlipidemia [35–37]. Further studies focusing on interval changes and links to outcomes with F₂-Isoprostane and 9-HETE are warranted.

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ABBREVIATIONS AND ACRONYMS

CAD

coronary artery disease

HODEs

hydroxy-octadecadienoic acids

HETE_s

	hydroxy-eicosatetraenoic acids
HPLC	high performance liquid chromatography
CRP	C-reactive protein
OR	odds ratio
NO	nitric oxide
Statin	3-hydroxy-3-methylglutaryl CoA Reductase inhibitor
EDTA	ethylenediaminetetraacetic acid
LC/ESI/MS/MS	on-line electrospray ionization tandem mass spectrometry
AA	arachidonic acid
m/z	mass-to-charge ratio
LA	linoleic acid

Figure 1.
Association between quartiles of 9-HETE/Arachidonate (mol/mol) and percent of patients with angiographically documented coronary artery disease.

Figure 2. Association between quartiles of F₂-Isoprostanes/Arachidonate (mol/mol) and percent of patients with angiographically documented coronary artery disease.

Table 1
Baseline Characteristics by Cardiovascular Disease Status*

Characteristic	Control/no CAD n = 50	Case/CAD n = 54	P-value
Age (yrs)	58 ± 12	65 ± 10	0.001
Males, (%)	23 (54)	33 (61)	0.12
Family history of CAD, n (%)	17 (34)	23 (43)	0.37
Diabetes mellitus, n (%)	10 (20)	18 (33)	0.13
Current smoker, n (%)	5 (10)	10 (18)	0.22
Aspirin, n (%)	24 (48)	38 (70)	0.02
Statins, n (%)	13 (26)	26 (48)	0.02
Angiotensin converting enzyme inhibitors, n (%)	17 (34)	18 (33)	0.94
B-Blockers, n (%)	15 (30)	33 (61)	0.001
Calcium channel blockers, n (%)	11 (22)	12 (22)	0.98
Nitrates, n (%)	11 (22)	22 (41)	0.04
Total cholesterol level (mg/dL)	181 ± 48	165 ± 35	0.06
High-density lipoprotein cholesterol (mg/dL)	50 ± 13	44 ± 15	0.01
Low-density lipoprotein cholesterol (mg/dL)	105 ± 37	96 ± 28	0.11
Triglycerides (mg/dL)	124 ± 66	126 ± 71	0.951
C-reactive protein (mg/L)	4.0 ± 5.8	7.4 ± 5.0	0.02

* Continuous measures are shown as mean ± standard deviation (SD), while categorical measures are shown as number and percentage with risk factor.

Table 2
Comparison of lipid peroxidation products Cardiovascular Disease Status*

Characteristic	Control/no CAD n = 50	Case/CAD n = 54	P-value
5-HETE (μmol/mol)	6.1 ± 5	6.7 ± 6	0.28
8-HETE (μmol/mol)	5.1 ± 4	4.8 ± 5	0.73
9-HETE (μmol/mol)	6.8 ± 4	8.7 ± 4	0.01
11-HETE (μmol/mol)	6.4 ± 3	7.3 ± 3	0.14
12-HETE (μmol/mol)	26 ± 14	20 ± 20	0.58
15-HETE (μmol/mol)	10 ± 6	14 ± 8	0.06
9-HODE (μmol/mol)	8.8 ± 4	9.1 ± 4	0.77
13-HODE (μmol/mol)	28 ± 13	32 ± 14	0.12
F ₂ -isoprostanes (mol/mol)	6.0 ± 3.0	9.0 ± 4.0	<0.001

* HODE (hydroxy-octadecadienoic acid); HETE (hydroxy-eicosatetraenoic acid)

Table 3Odds ratios of cardiovascular disease risk according to quartiles of 9-HETE and F₂-isoprostanes.

Model	1 st Quartile	2 nd Quartile	3 rd Quartile	4 th Quartile
9-HETE ((μmol/mol)				
Unadjusted [*]	1.0	2.1 (0.68–6.6)	3.6 (1.1–11.4)	3.4 (1.1–10.8)
Model 1 [†]	1.0	2.4 (0.75–7.9)	4.0 (1.2–13.2)	4.3 (1.3–14.3)
Model 2 [‡]	1.0	2.1 (0.64–7.2)	5.0 (1.4–17.5)	4.8 (1.3–17.1)
F ₂ -isoprostanes (mol/mol)				
Unadjusted	1.0	2.3 (0.73–7.4)	3.7 (1.2–11.9)	9.0 (2.6–31.8)
Model 3 [§]	1.0	2.4 (0.73–7.8)	3.9 (1.2–12.7)	8.9 (2.5–31.9)
Model 4 ^{//}	1.0	2.7 (0.79–8.9)	4.4 (1.3–15.1)	9.7 (2.6–36.9)

* Unadjusted odds ratios (ORs) and 95% confidence intervals (CIs). Each of the upper 3 quartiles for a given parameter were compared to the lowest quartiles.

[†] Model 1 consisted of Framingham Global Risk Score, and 9-HETE.

[‡] Model 2 consisted of Framingham Global Risk Score, usCRP, and 9-HETE.

[§] Model 3 consisted of Framingham Global Risk Score, and F₂-isoprostanes.

^{//} Model 4 consisted of Framingham Global Risk Score, usCRP and F₂-isoprostanes.