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Fatty acids and TxA₂ generation, in the absence of platelet-COX-1 activity

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Abstract

Background and aims—Omega-3 fatty acids suppress Thromboxane A₂ (TxA₂) generation via mechanisms independent to that of aspirin therapy. We sought to evaluate whether baseline omega-3 fatty acid levels influence arachidonic acid proven platelet-cyclooxygenase-1 (COX-1) independent TxA₂ generation (TxA₂ generation despite adequate aspirin use).

Methods and results—Subjects with acute myocardial infarction, stable CVD or at high risk for CVD, on adequate aspirin therapy were included in this study. Adequate aspirin action was defined as complete inhibition of platelet-COX-1 activity as assessed by <10% change in light transmission aggregometry to 1 mmol/L arachidonic acid. TxA₂ production was measured via liquid chromatography–tandem mass spectrometry for the stable TxA₂ metabolite 11-dehydro-thromboxane B₂ (UTxB₂) in urine. The relationship between baseline fatty acids, demographics and UTxB₂ were evaluated. Baseline omega-3 fatty acid levels were not associated with UTxB₂ concentration. However, smoking was associated with UTxB₂ in this study.

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Disclosures

Dr. DeFilippis serves on a study adjudication committee for Radiometer America to determine clinical truth (diagnosis of study participants). Dr. Allan S. Jaffe is a consultant for Alere, Beckman–Coulter, Radiometer, Critical Diagnostics and Amgen.

Conclusion—Baseline omega-3 fatty acid levels do not influence TxA₂ generation inpatients with or at high risk for CVD receiving adequate aspirin therapy. The association of smoking and TxA₂ generation, in the absence of platelet COX-1 activity, among aspirin treated patients warrants further study.

Keywords

Omega-3 fatty acids; Thromboxane; Aspirin resistance; Platelet activation; Smoking

Introduction

Acute ischemic heart disease is a leading cause of death in the United States and is most often the result of coronary atherothrombosis. Platelet aggregation is fundamental to the atherothrombotic processes. Prostaglandin thromboxane A₂ (TxA₂) belongs to one of several pathways that are pivotal in stimulating platelet aggregation [1]. TxA₂ is generated via release of the platelet membrane omega-6 fatty acid arachidonic acid, which ultimately is the substrate for aspirin's therapeutic target, cyclooxygenase-1 (COX-1) [2]. It has been demonstrated that platelets have the capacity to increase the production of TxA₂ several thousand fold above baseline production; making it an attractive measure to evaluate platelet activation [1].

Baseline concentrations of long-chain omega-3 fatty acids are inversely associated with risk of sudden cardiac death and omega-3 fatty acid therapy has been shown to reduce cardiovascular events in many but not all studies [3,4]. Reduced production of TxA₂ is one mechanism by which omega-3 fatty acids may reduce cardiovascular events. The three most biologically active omega-3 fatty acids are eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and alpha-linolenic acid (ALA). ALA can be enzymatically converted to EPA and then DHA [4]. These omega-3 fatty acids can displace omega-6 fatty acid cell membrane stores (arachidonic acid) needed to produce TxA₂ and act as competitors for the same metabolic pathways [5]. In vitro studies of human platelets demonstrate reductions in TxA₂ production in the presence of omega-3 fatty acids [6] and some have hypothesized that individuals with higher membrane omega-3 content will produce less platelet activating TxA₂ than individuals with lower membrane omega-3 levels. The antiplatelet effect of omega-3 fatty acids was supported by a study of 62 patients with stable cardiovascular disease and a laboratory assessment consistent with "aspirin resistance". Omega-3 fatty acids supplementation reduced the incidence of "aspirin resistance" by 80% in this population [7]. Additionally, a decline in urine 11-dehydro thromboxane B₂ (UTXB₂), a stable metabolite of TxA₂, following increasing intake of omega-3 fatty acids has been observed [8]. EPA has been shown to reduce UTXB₂ production beyond that achieved by aspirin alone in healthy volunteers [9].

Prior work has demonstrated that TxA₂ production may occur despite the complete suppression of platelet COX-1 activity with aspirin therapy [10,11]. In patients with stable CAD treated with adequate doses of aspirin, TxA₂ production is correlated with the occurrence of future cardiovascular events [12]. Given the ability of omega-3 fatty acids to suppress TxA₂ generation via mechanisms independent to that of aspirin therapy, baseline

omega-3 membrane fatty acid content may be one determinant of platelet-COX-1 independent TxA₂ production.

In the present study, we sought to evaluate the relationship between COX-1 independent TxA₂ generation and omega-3 fatty acid levels. Appropriate aspirin action was required by assessment of platelet COX-1 activity via arachidonic acid aggregometry. The cohort included individuals with both stable and active CAD. We measured TxA₂ production via a liquid chromatography–tandem mass spectrometry (LC-MS/MS) assay for the stable TxA₂ metabolite 11-dehydro-thromboxane B₂ in urine (UTxB₂). Baseline omega-3 fatty acid levels were assessed by measurement of fatty acid content of the erythrocyte lipid bilayer [13–15].

Methods

Study population

Participants were recruited from a single academic medical center between September 2008 and August 2010. Those enrolled met the following criteria: 1) >18 yr of age, 2) scheduled for coronary angiography within 48 h, and 3) reported ingesting an adequate aspirin dose (>160 mg on the prior calendar day but within the last 24 h of presentation, or >75 mg/d for the last 7 days). Subjects with a platelet count <100,000/mm³, unable to produce urine and those with a major vascular event (cardiac catheterization, stroke, myocardial infarction) during the past 12 weeks or coronary bypass surgery in the prior year were excluded from enrollment.

Potential study participants were identified by treating physicians. All subjects referred to the study were evaluated and enrolled if they met the study inclusion/exclusion criteria. The most common inclusion criteria failed to be met was confirmation of adequate aspirin therapy or a sufficient time period for enrollment prior to coronary angiography procedure. Measurements of UTxB₂ and red blood cell membrane fatty acid content were made prior to cardiac catheterization (enrollment). The study was approved by the Johns Hopkins Institutional Review Board (IRB). All patients provided written informed consent.

History, physical exam, and ECG data

A single study physician interviewed all participants and finalized history, physical exam, and ECG data prior to any measurement of UTxB₂. The study interview included specific questioning with regard to the timing, dose, and frequency of aspirin and NSAID use in the 7 days prior to enrollment and the nature and timing of anginal symptoms. The medical record was used to aid in collection of pertinent medical history (i.e., timing of medication administration). Standard laboratory data (troponin, creatinine, blood cell and platelet counts) were obtained from the medical record.

Biochemical data

A urine specimen was collected from each participant prior to cardiac catheterization, and specimens were centrifuged, aliquotted, and stored at –70 °C until analyzed. Metabolites of TxA₂ were assessed with a quantitative analysis of urine TxB₂ using a novel LC-MS/MS

assay at the Mayo Clinic, Mayo Medical Laboratories, Rochester, MN. Urine samples were adjusted to a pH of 2.0 ± 0.2 and incubated for 3 h at ambient temperature to force all the urinary thromboxane into the closed ring form that is recognized by the LC-MS/MS method. After addition of d4 internal standard (d4-11-dTxB₂) to each sample, the samples were positive pressure-filtered, and the 11-dTxB₂ was separated from the urine matrix via turboflow online extraction. A Cyclone MAX anion-exchange column (Thermo Fisher Scientific, Franklin, MA) was used for the extraction, and a Waters Xbridge C8 (Waters Corporation, Milford, MA) was used for separation from other prostaglandins. From this column, the samples were transferred to an API 5000 MS/MS for instrumental analysis. Intra-assay precision was 4.4% and 2.9% for low (412 pg/mL)- and high (2826 pg/mL)-level urine pools, respectively. Inter-assay precision was 9.8% and 11.9% for low (624 pg/mL)- and high (4782 pg/mL)-level urine pools, respectively. All measurements were performed in duplicate, and the average TxB₂ result is reported relative to urinary creatinine. Healthy individuals taking aspirin typically have 11-dehydro-TxB₂ concentrations < 500 pg/mg creatinine when measured by these methods [16].

Erythrocyte membrane total lipid fatty acids were derivatized to their pentafluoro-benzyl bromide esters which were separated and identified by negative ion gas chromatography mass spectrometry as previously described [17]. Coefficients of variance of the fatty acids of interest (arachidonic acid, total omega-3/6 fatty acids) are all <13% in this laboratory. All fatty acid measurements were normalized to total membrane fatty acid content. Therefore fatty acid levels represent the proportion (percent) of total membrane fatty acid content.

Assessment of platelet COX-1 activity

Platelet COX-1 activity in response to 1 mmol/L arachidonic acid was measured by light transmission aggregometry using a Chrono-Log Model 700 aggregometer in accordance with established methodology [18,19]. Changes in absorbance were converted to percent aggregation by reference to the absorbances of platelet-rich plasma and platelet-poor plasma. A positive test for aggregation, indicating incomplete inhibition of COX-1, was defined as a >10% increase in light transmission in response to 1 mmol/L arachidonic acid [20]. Control samples from individuals not exposed to any aspirin, NSAID, or anti-platelet drug for the previous 7 days were run using the same reagents and methodology within 24 h of study subject testing. For the assay to be considered valid, control samples had to show >80% aggregated in response to <1 mmol/L arachidonic acid.

Statistical analysis

For this statistical analysis, all subjects with incomplete inhibition of COX-1 were excluded. Regression analysis using UTxB₂ measurement as the dependent variable and each fatty acid, demographic and physical characteristics as the independent variable was performed. A log 10 transformation was used for the UTxB₂ measurements in the regression analysis. Univariate analysis was also performed on each of the fatty acids absolute concentration (without standardizing to total membrane fatty acid content). Multivariable regression results using the log 10 transform of UTxB₂ as the dependent variable and demographic and fatty acid characteristics found significant in univariable regression ($p \leq 0.05$) was performed. All possible two-way interactions of these main effects were explored separately

along with the significant main effects in multivariable regression. Interactions with p -values <0.05 were included in a multivariable regression model along with the main effects. Interaction effects with p -values >0.05 were then dropped from the model. Finally, main effects with p -values >0.05 and not part of the remaining significant interaction effects were also dropped from the model. Two-way interaction terms were explored individually and those with p -values less than or equal to 0.05 were included in the multivariable regression.

Results

A total of 54 subjects were enrolled. We excluded 10 subjects due to inadequate aspirin therapy as determined by examination of medical records or $>10\%$ platelet aggregation in response to 1 mmol/L arachidonic acid, resulting in 44 subjects for this analysis. This included simultaneous measurements of UTxB₂ and platelet fatty acid profile on all subjects.

The mean age was 66 years with a high prevalence of multiple cardiovascular risk factors and coronary artery disease (50% coronary stenosis via coronary angiography) in greater than 75% of patients at the time of enrollment (Table 1).

Baseline, mean, median and range of UTxB₂ concentrations for the entire cohort and stratified among multiple baseline characteristics is summarized in Table 2. Only smoking had a statistically significant impact on UTxB₂ concentrations, with current smokers having a higher level than those who were not current smokers.

Table 3 details the mean, median and range of membrane fatty acid concentrations as assessed by mass spectrometry, including arachidonic acid (AA), alpha linolenic acid (ALA), eicosapentaenoic acid (EPA), docosahexanoic acid (DHA), total omega 6 acid and total omega 3 acid.

No fatty acid was associated with UTxB₂ when assessed as a proportion of total membrane fatty acid content (Table 4). Further univariable and multivariable analysis of association of Log₁₀ UTxB₂ and the fatty acid absolute concentration found no consistent associations (data not shown).

Discussion

This study failed to identify a relationship between base-line omega-3 fatty acids and TxA₂ generation, in the absence of platelet COX-1 activity, in a cohort of individuals with arachidonic acid aggregometry proven aspirin action and a high incidence of cardiovascular disease/cardiovascular disease risk factors.

Over the last 3 decades, epidemiologic and experimental data have provided evidence for a beneficial effect of omega-3 fatty acids in the prevention of CVD [4]. Omega-3 fatty acids have been shown to have a favorable impact on multiple factors related to CVD, including reduced platelet activation [4]. It is known that omega-3 fatty acids can displace omega-6 fatty acid stores needed to produce TxA₂, in cell membranes and act as competitors for the same metabolic pathways [5]. Furthermore, in vitro studies of human platelets demonstrate

reductions in TxA₂ production in the presence of omega-3 fatty acids [6]. Recent work has demonstrated that TxA₂ production may persist despite appropriate aspirin action in acute cardiac patients [10]. We postulated that differences in the production of TxA₂, despite adequate aspirin action, may be related to differences in available substrate omega-3 versus omega-6 fatty acids. However, multiple measures of membrane fatty acid content failed to show a consistent association with platelet-COX-1 independent TxA₂ levels, as assessed by LC-MS/MS measurement of UTxB₂. The mechanism by which platelet-COX-1 independent TxA₂ is generated is not well understood but this study suggests that baseline fatty acid content is not an important determinant.

An examination of subject demographics demonstrated an association between smoking and TxA₂ levels, in the absence of platelet COX-1 activity. Multiple studies have demonstrated an association between tobacco smoke and increased levels of thromboxane in patients not taking aspirin [21–23]. In patients taking aspirin, tobacco smoking is also associated with higher thromboxane levels and although aspirin therapy reduces thromboxane levels in smokers, it is not reduced to the same level as non-smoking controls on aspirin therapy [24,25]. Unlike COX-1, COX-2 is significantly augmented by inflammatory stimuli, including cigarette smoking, and therefore may account for the positive association between UTxB₂ and smoking in our study [22,24]. Additional evidence that increase TxA₂ generation among smokers is mediated via COX-2 was demonstrated in a study which revealed a reduction in TxA₂ generation with the COX-2 specific inhibitor, rofecoxib, among smokers but not non-smokers [26]. The current study differs from the afore mentioned studies because the current study is the only study to establish appropriate aspirin action with an objective biochemical test –eliminating confounding from reduced aspirin compliance. Our results demonstrate an association between tobacco smoking and higher thromboxane levels in patients with arachidonic acid aggregometry proven aspirin induced platelet COX-1 inhibition. Such findings may explain one mechanism by which tobacco smokers are at increased risk for cardiovascular events and therefore warrants further investigation.

Limitations

Although arachidonic acid-induced platelet aggregation is the current standard for evaluating platelet COX-1 activity [20], it is possible that this technique is without adequately sensitive for detecting reduced, but not completely absent, platelet COX-1 activity. TxA₂ was not measured directly in this study. TxA₂ has a short circulatory half life of 30 s, but its stable inactive hydration product, TxB₂, is further metabolized to produce two major stable metabolites 2,3-dinor TxB₂ and 11-dehydro-TxB₂ (half-life of 15 & 45 min respectfully) which are subsequently cleared in the urine. Plasma measures of TxA₂ and its metabolite, TxB₂, are confounded by platelet activation leading to in vitro release of TxA₂/TxB₂, inherent to the process of phlebotomy. Intra-renal TxB₂ production confounds the measure of urine TxB₂ as a reflection of systemic TxB₂ production and 2,3-dinor TxB₂ excretion into the urine is non-linear. However, the most abundant and stable metabolite of TxB₂, 11-dehydro-TxB₂, is excreted into the urine linearly, over a range greater than calculated rates of endogenous TxA₂ generation. Therefore the 11-dehydro-TxB₂ measured in the urine (UTxB₂), reflects phasic TxA₂ generation and platelet activation in humans and

was used to assess TxA₂ generation in this study [27,28]. Quantitative liquid chromatography–tandem mass spectrometric analysis is the gold standard for measuring UTxB₂ [29]. A second limitation is the limited sample size precluded detailed multivariable analyses/complete investigation of potential confounders and limited our power to demonstrate a difference in UTxB₂, if one truly exists in these cohorts. While many factors (i.e. acute MI) are associated with TxA₂ generation in the absence of aspirin therapy, we know of no data demonstrating such a relationship in subjects on proven adequate aspirin therapy. We evaluated multiple factors, including acute MI and history of CVD and statin use at the time of sample collection in a univariate analysis (Table 2) –only smoking was related to aspirin independent TxA₂ generation. Additionally, 88.6% (39 of 44) of subjects in this study had TxB₂ <500 pg/mg creatinine, consistent with a normal response to aspirin. One might expect a greater impact from baseline fatty acid levels on TxB₂, in a study targeting only individuals who have an abnormal response to aspirin TxB₂ (<500 pg/mg creatinine). This question warrants further study.

Conclusions

Baseline omega-3 fatty acid levels do not have an effect on TxA₂ production in the presence of arachidonic acid proven adequate aspirin therapy. The association of tobacco smoke exposure and platelet-COX-1 independent TxA₂ generation warrants further study.

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Table 1

Distribution of patient characteristics.

Variables	Total N (%)
<i>Gender</i>	
Male	31 (70.5)
Female	13 (29.5)
<i>Race</i>	
Caucasian	29 (65.9)
Other	15 (34.1)
<i>Smoking history</i>	
Never	20 (45.5)
Current	4 (9.1)
Former	20 (45.5)
History of diabetes	11 (25.0)
History of hypertension	36 (81.8)
History of dyslipidemia	35 (79.5)
History of MI, CABG, or PCI	20 (45.5)
History of stroke, CEA, PAD, or AAA	6 (13.6)
History of congestive heart failure	9 (20.5)
<i>Statin use at time of enrollment</i>	
Yes (%)	11 (25.0)
No (%)	33 (75.0)
Obstructive CAD on enrollment angiogram ^a	35 (79.5)
<i>Final diagnosis of acute MI^b</i>	
Yes	22 (50.0)
No	22 (50.0)
<i>Numeric variables</i>	
Age mean (SD)	66.4 (12.7)
BMI mean (SD)	30.0 (8.0)
SBP mean (SD)	137.7 (23.6)
DBP mean (SD)	75.4 (14.2)
Arachidonic acid aggregometry at enrollment mean (SD)	3.0 (2.0)

^a No coronary stenosis >50% in 9 subjects.^b Clinical attending WHO myocardial infarction diagnosis.

Table 2

Association of Patient Characteristics with UT_xB₂ (pg/mg creatinine) in the absence of platelet-COX-1 activity.

Characteristic	Category	Mean(SD)	Med(range)	P
Total		325(423)	255(69–2829)	–
Gender	Male	325(495)	222(69–2829)	0.159
	Female	327(122)	350(106–504)	
Race	Cauc.	335(499)	255(69–2829)	0.642
	Other	306(200)	277(83–739)	
Smoking	Never	246(168)	244(69–692)	0.041
	Current	966(1248)	426(184–2829)	0.017*
	Former	273(164)	244(74–739)	
Diabetes	Yes	296(188)	312(70–692)	0.938
	No	336(480)	238(69–2829)	
Hypertension	Yes	278(166)	261(70–739)	0.899
	No	535(937)	194(69–2829)	
Dyslipidemia	Yes	265(176)	244(69–739)	0.084
	No	641(968)	321(144–2829)	
History of MI, CABG, PCI	Yes	277(165)	247(74–692)	0.975
	No	367(560)	255(69–2829)	
History of stroke, PAD or CEA	Yes	342(190)	321(74–544)	0.555
	No	323(446)	238(69–2829)	
History of CHF	Yes	317(229)	315(74–739)	0.896
	No	331(465)	249(69–2829)	
No obstructive CAD ^a	Yes	536(931)	249(70–2829)	0.712
	No	277(172)	261(69–739)	
Acute MI	Yes	297(167)	312(69–739)	0.521
	No	356(587)	244(70–2829)	
Statin use at enrollment	Yes	243 (121)	233 (83–447)	0.652
	No	354 (484)	258 (69–2829)	
		<i>b</i> ₁	<i>P</i>	
Age		0.0036	0.388	
BMI		0.0033	0.675	
SBP		0.0017	0.435	
DBP		–0.0017	0.641	
AAA		–0.032	0.211	

* *P*-value for current vs. former/never; Log₁₀ UT_xB₂ used for *p*-values.

MI = Myocardial infarction; CABG = Coronary artery bypass graft; PCI = Percutaneous coronary intervention; PAD = Peripheral artery disease; CEA = Carotid endarterectomy; CHF = Congestive heart failure; CAD = Coronary artery disease; BMI = Body mass index; SBP = Systolic blood pressure; DBP = Diastolic blood pressure; AAA = Arachidonic acid aggregometry.

Bold text notes those variables which have met statistical significance at the 0.05 levels.

^aNo coronary stenosis >50%.

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Table 3

Distribution of fatty acids.

Proportion (percent) of total membrane fatty acid content	N	Mean	SD	Median	Min	Max
AA	44	12.8	1.4	13.1	8.6	16.0
ALA	44	0.1	<0.1	0.1	<0.1	0.2
EPA	44	0.5	0.5	0.4	0.1	3.4
DHA	44	3.3	1.1	3.0	1.4	6.9
Omega 6	44	27.1	2.4	27.4	18.5	31.8
Omega 3	44	5.6	1.8	5.2	3.0	13.7

FA – Fatty acid.

Table 4Association of Log 10 UT_xB₂ and fatty acids.

Fatty acid	<i>b</i> ₁	<i>P</i>	<i>r</i>
AA	0.219	0.952	0.009
ALA	372.06	0.101	0.254
EPA	6.556	0.53	0.098
DHA	1.616	0.732	0.054
Omega 6	2.486	0.248	0.18
Omega 3	1.635	0.563	0.091
EPA + DHA	1.588	0.646	0.072

FA-Fatty acid; *b*₁ – regression coefficient based on log 10 UT_xB₂ MS; *P* – *P*-value based on log 10 UT_xB₂; *r* – Pearson correlation coefficient.