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Diminished Omega-3 Fatty Acids are Associated with Carotid Plaques from Neurologically Symptomatic Patients: Implications for Carotid Interventions

Hernan A. Bazan^{1,2,†}, Yan Lu, MD³, Deepu Thoppil, MD⁴, Tamara N. Fitzgerald, MD, PhD⁵, Song Hong, PhD³, and Alan Dardik, MD, PhD⁵

¹Department of Surgery, Section of Vascular Surgery, Louisiana State University Health Sciences Center, New Orleans, LA

²Department of Pharmacology and Experimental Therapeutics, Louisiana State University Health Sciences Center, New Orleans, LA

³Neuroscience Center, Louisiana State University Health Sciences Center, New Orleans, LA

⁴Gene Therapy Center, Louisiana State University Health Sciences Center, New Orleans, LA

⁵Department of Surgery, Section of Vascular Surgery, Yale University School of Medicine, New Haven, CT and VA Connecticut Healthcare System, West Haven, CT

Abstract

The omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are prevalent in fish oil and their cardioprotective effects are thought to be mediated by anti-inflammatory mechanisms. The aim of this study is to determine whether omega-3 fatty acids are associated with carotid plaques from neurologically symptomatic patients. Plaques were obtained from 41 patients (mean age 62 [44 – 84]; 24-asymptomatic, 17-symptomatic). Intra-plaque lipids were assessed with mass spectrometry. Compared to asymptomatic patients, significantly diminished omega-3 fatty acids DHA (545.8 ± 98 ng/g vs. 270.7 ± 19.6 ng/g, $p=0.0096$) and EPA (385.9 ± 68 ng/g vs. 216.4 ± 17.6 ng/g, $p=0.0189$) were found in carotid plaques from neurologically symptomatic patients. However, no differences were found in the levels of the omega-6 fatty acid arachidonic acid ($p=0.2003$). Immunohistochemistry and ELISA analysis ($CD68^+$ cells, 0.461 ± 0.04 vs. 0.312 ± 0.03 , $p=0.003$) demonstrated an increased inflammatory infiltrate in plaques from neurologically symptomatic, compared to asymptomatic, patients. Carotid plaques from neurologically symptomatic patients are inflammatory and have decreased intra-plaque levels of omega-3 fatty acids. Future trials will determine whether interventions that increase omega-3 fatty acid incorporation into carotid plaques prevent stroke and improve the safety of carotid interventions.

Keywords

carotid; plaque; instability; omega-3; inflammation

†Correspondence: Hernan A. Bazan; MD, LSUHSC – New Orleans, School of Medicine, Section of Vascular Surgery, 1542 Tulane Ave, Room 733, New Orleans, LA 70112, Telephone: (504) 568-4750, Fax: (504) 5684730, hbazan@lsuhsc.edu.

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Introduction

Increasing evidence suggests that consumption of long-chain omega-3 polyunsaturated fatty acids (**PUFAs**) protects against cardiovascular disease, especially fatal myocardial infarction and sudden cardiac death [1],[2,3]. Since humans cannot synthesize the omega-3 PUFA, eicosapentaenoic acid (**EPA**) and docosahexaenoic acid (**DHA**), intake comes predominantly from consumption of fish or fish-oil. The cardiovascular protection of omega-3 PUFAs may occur independently of their lipid-lowering effect[4,5]. Omega-3 fatty acids have anti-inflammatory[6] and anti-arrhythmic effects[5,7], and may improve endothelial function[8].

Atherosclerotic plaques arise in the vessel intima and are thought to be a result of cholesterol deposition, hemodynamic strain, and inflammation[9]. Rupture of the fibrous cap leads to a transition from a stable or asymptomatic to an unstable or symptomatic atherosclerotic plaque. This plaque rupture results in clinically relevant sequelae; in the coronary bed, atherosclerotic plaque rupture leads to a myocardial ischemic event and, in the carotid artery, atherosclerotic plaque rupture leads to an ocular or cerebral ischemic event. These latter events manifest as either amaurosis fugax, transient ischemic attacks, or stroke. The mechanisms leading to atherosclerotic plaque rupture are not completely understood and various inflammatory molecules have been hypothesized to play a role[10]. One set of molecules purported to be involved with carotid plaque instability are omega-3 PUFAs[11]. Thies and colleagues demonstrated that supplementation with fish oil can lead to PUFAs incorporation into carotid atherosclerotic plaques, thickening the fibrous caps, potentially stabilizing them[11]. Patients undergoing carotid endarterectomy (**CEA**) are ideally suited for studying the biochemical mechanisms involved in the transition from an asymptomatic to a symptomatic atherosclerotic plaque primarily due to the ease of harvesting the tissue samples.

One potential mechanism leading to decreased cardiovascular events is via atherosclerotic plaque stabilization through the anti-inflammatory effects of omega-3 PUFAs. We sought to determine whether patients with advanced carotid plaques, undergoing CEA, had differential levels of omega-3 PUFAs incorporated into the plaques, and whether these levels of PUFAs were correlated with plaque symptomatology and inflammation.

Methods

Study Design

Patients with at least 50% internal carotid artery stenosis undergoing carotid endarterectomy were included in this study. Informed consent was obtained after approval through the Louisiana State University Health Sciences Center Institutional Review Board and the Veterans Affairs Connecticut Healthcare System. For each enrolling patient, the age, sex, history of cardiac disease, chronic renal insufficiency (defined as a serum creatinine ≥ 1.6 mg/dL), diabetes, hypertension, and history of tobacco use were recorded, as well as current drug treatment (antiplatelet, oral hypoglycemic, antihypertensive, or lipid-lowering). Any previous ipsilateral symptoms were also recorded lasting less than six months. Carotid plaque stability was determined on the basis of clinical criteria. Asymptomatic patients with high-grade carotid stenosis ($\geq 75\%$ internal carotid stenosis based on duplex ultrasound imaging) were included in the asymptomatic carotid atherosclerotic plaque group. Patients presenting with symptoms of temporary or partial complete loss of sight, a transient ischemic attack, and/or an established stroke with good neurologic recovery in the previous six months with $\geq 50\%$ stenosis and deemed safe by the operating surgeon to undergo carotid endarterectomy were included as symptomatic carotid plaques. Harvested carotid atherosclerotic plaques were bisected and one portion was placed into a conical tube containing zinc formalin for immunohistochemical analysis and the other is cut in half; this was further divided into two sections and half placed into a tube containing RNAlater (Applied Biosystems/Ambion, Austin, TX), for RNA

and protein analysis for future experiments, and the other half in buffered saline for lipid extraction.

Lipid extraction and mass spectrometry

Endarterectomy specimens from carotid atherosclerotic plaques were harvested in buffered 0.9% normal saline and stored in -80°C until analysis. Deuterium-labeled internal standards [50 ng of each, d_4 -prostaglandin D_2 (**PGD₂**) and d_5 -DHA] are added to each plaque sample to determine the extraction recoveries (typically $>80\%$) of the lipid mediators. The plaque was homogenized and sonicated in 2 ml methanol (pH 3.5). The extract was centrifuged and supernatant was collected. Each pellet was then extracted with Chloroform: Methanol (2:1, v/v, 2 ml) twice more. Part of the pooled supernatants for each sample was cleaned up with carbon-18 solid phase extraction; the cleaned extracts were reconstituted into methanol (**MeOH**) and analyzed via liquid chromatography-ultraviolet spectrometry-tandem mass spectrometry (**LC-UV-MS/MS**) for lipidomic profiles of eicosanoids and docosanoids. In order to analyze the fatty acid composition of complex lipids (e.g., phospholipids), hydrolysis of the esterified fatty acids was conducted in another portion of the extract. This quantitative hydrolysis produced free fatty acids suitable for mass spectrometric analysis. The procedures were as follows: 1) lipid extract was suspended in 50 microliters (μL) of methanol, 2) 8 μL of 1 M sodium hydroxide (**NaOH**) and 42 μL H_2O was added 3) nitrous gas (**N₂**) flush was used and specimen incubated at 42°C for 3 h, 4) 100 μL H_2O was added, adjusted pH to 4 with 0.05 M hydrochloric acid (**HCl**), aqueous phase extracted with Hexane: Isopropanol (3:2 V/V) 2 mL, vortex 20 s, 5) centrifuged at 3000 rpm for 5 min, removed organic phase from top of the aqueous phase, and washed with 1 mL solvent mixture, 6) finally, dry organic extracts were resuspend in methanol for LC-UV-MS/MS analysis.

Protein extraction and analysis

Tissue frozen at -80°C was slowly defrosted on ice. The tissues were then cut longitudinally through the center of the lesion into a portion approximately 0.1g. The tissue was then placed on ice for homogenization and homogenized using 1.5 ml tubes containing a grinding resin using a plastic pestle (MicroRotor for Lysis Kit [Mammal], BioRad Laboratories, Hercules, CA), as per the manufacturer's recommendations. Protein concentration was determined using the RC/DC Protein Assay Kit (BioRad Laboratories, Hercules, CA) and read at an optical density of 650 nm. CD68 was assayed using Duo Set enzyme linked immunosorbent assay (**ELISA**) Kits (R&D Systems Inc., Minneapolis, MN). A 96-well polystyrene ELISA enhanced plate (BD Biosciences, San Jose, CA) were coated with a capture antibody, incubated overnight at 4°C , and the next day control proteins were diluted in PBS, as per the standard protocol. Patient protein samples were diluted in PBS to 25 $\mu\text{g}/\text{ml}$, 6.25 $\mu\text{g}/\text{ml}$ and 0.78 $\mu\text{g}/\text{ml}$. The diluted samples were then added to the plate at a volume of 100 μL per well. After two hours the plates were washed 4 times with PBS-Tween-20, according to the directions provided. All subsequent steps were as per the standard protocol. Color was developed using 100 μL of TMB ELISA Substrate (Pierce) and 50 μL of sulfuric acid. The optical density was then read on a standard plate reader (BioRad Laboratories, Hercules, CA). Relative protein concentrations were then determined.

Histology and immunohistochemistry

Plaques were harvested in zinc-buffered formalin for histology and immunohistochemical analysis. Following fixation, all samples were dissected into five cross-sections ranging from 0.3 to 0.5 mm in greatest dimension stemming from the grossly identified center of each plaque. The middle segment was used for all histological analyses. The remaining fragments were saved for future studies. Selected tissue segments were subject to paraffin processing and embedding followed by brief, surface decalcification in Decalcifier II solution (Surgipath,

Richmond, IL) and serial sectioning at 4 μ m increments onto glass slides. One set of slides from each plaque was stained with hematoxylin and eosin (H&E) and the rest were pretreated for indirect immunofluorescence. Briefly, slides were heated at 60°C for 45 minutes, deparaffinized in xylene, and rehydrated in graded ethanol series to water. Next, samples were pre-treated with Proteinase K and sections washed in 0.5% Tween-20 in Tris-buffered saline. Tissues were treated for non-specific protein binding and endogenous autofluorescence with 5% goat serum diluted in 1% BSA for 15 minutes and Image-iT FX (Molecular Probes, Eugene, OR), respectively. Following washes, sections were incubated overnight at 4°C with mouse anti-CD68 (4.7 μ g/mL, Dako, Carpinteria, CA). Negative controls were generated by omission of the primary antibody. Goat-derived antibodies against each primary's source species' Ig tagged with Alexa 568 (4 μ g/mL; Molecular Probes, Eugene, OR) for 30 minutes at room temperature were then applied. The nuclear counterstain 4',6-diamidino-2-phenylindole (Dapi) was applied at 300mM for 5 minutes. Histopathology analysis of the H&E-stained slides was performed under a Nikon E600 transmitted light microscope (Diagnostic Instruments, Sterling Heights, MI). Matching immunofluorescent sections were imaged with a Leica DMRXA upright, epifluorescent microscope with filters specific for Dapi (D350/50 $\times$; 400DCLP; D460/50m). Simultaneous image capture of all probes; background correction; autofluorescence subtraction using a green-shifted fluorescence filter cube; and quantitative analysis were performed with Slidebook software. Measurements were acquired through mask subsampling over steady ranges of probe intensities across samples followed by calculation of area occupied by each immunological marker in μ m².

Statistical analysis

The effects of omega-3 and omega-6 fatty acids on carotid plaque stability were analyzed by univariable analysis; p-values were calculated using Chi-square or Fischer's exact test (GraphPad Prism 5.01, GraphPad Software Inc, La Jolla, CA). All tests were two-tailed and p-values $\leq$ 0.05 were considered statistically significant (Statview 5.0, SAS Institute, Cary, NC).

Results

Carotid atherosclerotic plaques were obtained from 41 patients (35 male; mean age 62, ages 44 - 84); 24 (59%) were considered asymptomatic and 17 (41%) symptomatic (Table I). There were no significant differences in the distribution of patient comorbidities, including cardiac disease, chronic renal insufficiency, diabetes mellitus, hypertension, and tobacco use; no differences in overall atherosclerotic burden could be assessed between the two groups. Of those patients with symptomatic carotid atherosclerotic plaques, 11% presented with temporary partial or complete loss of sight (amaurosis fugax), 53% with transient ischemic attacks, and 44% with an established stroke but good neurologic recovery (Table I).

Since symptomatic plaque morphology is thought to be related to increased intra-plaque inflammation[9], we examined whether carotid plaques from neurologically symptomatic patients have morphological signs of inflammation. Histology revealed the architectural distortion in carotid plaques from neurologically symptomatic patients compared to stable ones (Figures 1A). Immunohistochemistry for CD68 demonstrated increased staining in carotid plaques from neurologically symptomatic patients, directly showing increased inflammatory cells in symptomatic plaques (Figure 1B). The mean positive area for CD68+ antibody staining over the total tissue area demonstrated an increased expression of CD68 cells in symptomatic compared to asymptomatic carotid plaques ($P = .009$). This was confirmed quantitatively, utilizing ELISA; carotid plaques from neurologically symptomatic patients have increased expression of CD68⁺ cells compared to stable plaques, $P = .003$ (Figure 1C). These results

show that carotid plaques from neurologically symptomatic patients have histological and immunological correlation with increased inflammation.

Since the omega-3 fatty acids DHA and EPA are thought to reduce plaque inflammation, we examined whether symptomatic plaques have reduced levels of these omega-3 PUFAs. Compared to asymptomatic carotid atherosclerotic plaques, significantly lower levels of the omega-3 fatty acids DHA (545.8 ± 98 ng/g vs. 270.7 ± 19.6 ng/g, $P = .019$) were found in symptomatic carotid plaques (Figure 2A). The omega-3 fatty acid EPA was similarly decreased in carotid plaques from neurologically symptomatic patients compared to asymptomatic ones (385.9 ± 68 ng/g vs. 216.4 ± 17.6 ng/g, $P = .038$), respectively (Figure 2B). However, no significant differences were found in the plaque content of the omega-6 fatty acid AA between asymptomatic and symptomatic plaques (1644 ± 356 ng/g vs. 2611 ± 1207 , $P = .40$, respectively (figure 2C). The content of various other lipids, including monohydroxyeicosatetraenoic acids (HETEs; 5HETE, 11HETE, 12HETE, and 15HETE) and monohydroxy eicosapentaenoic acids (HEPEs; 5HEPE, 12HEPE, 15HEPE, and 18HEPE) did not differ significantly between asymptomatic and symptomatic carotid atherosclerotic plaques (data not shown).

Discussion

We demonstrate that carotid plaques from neurologically symptomatic patients are more inflammatory and composed of diminished omega-3, but not omega-6, polyunsaturated fatty acids compared to asymptomatic carotid plaques. We found a significant correlation between symptomatic carotid atherosclerotic plaques, reduced omega-3 PUFA content, and increased inflammation, as evidenced by CD68+ staining and quantification. Though not statistically significant, we observed a trend towards an increased content of the omega-6 fatty acid AA in symptomatic plaques, consistent with others' suggestion that this pathogenic lipid may be involved in plaque instability[11].

PUFAs are fatty acids containing two or more double bonds in the acyl chain. The two main types of PUFAs are omega-3 and omega-6, which are distinguished by the position of the double bond closest to the methyl end of the acyl chain. In omega-6 fatty acids, the double bond is on carbon number 6 from methyl end, whereas in omega-3 fatty acids it is on carbon number 3 from methyl end. Since humans cannot synthesize EPA or DHA, the main sources of omega-3 PUFAs are fish; alternatively, DHA and EPA can be synthesized to a limited extent from dietary omega-3 linolenic acid, which main source is vegetable oil. Omega-3 PUFAs are essential substances for the development and function of various human organs, including the retina and central nervous system[12].

The omega-3 PUFAs are thought to have several biological functions which attenuate thrombosis and inflammation, including reduction of platelet aggregation[13], promotion of vasodilation[14], and plaque-stabilization[11,15]. These pleiotropic effects of omega-3 PUFAs on cardiovascular events are of potential therapeutic importance. That omega-3 PUFAs are cardioprotective is further underscored by a recent, large randomized, open-label trial demonstrating decreased coronary events in hypercholesterolemic Japanese patients treated with EPA (1800 mg per day), irrespective of low-density lipoprotein (LDL) cholesterol concentration[16]. Sub-analysis of patients having sustained a stroke demonstrated a potential benefit of EPA supplementation on stroke recurrence over a 5 year period[17]. In this secondary prevention subgroup, 457 of the stroke patients received a placebo (control) supplement while 485 stroke patients received daily supplementation with 1800 mg/day EPA over a 5 year period; all patients were maintained on statin therapy. Stroke recurrence occurred in 10.5% of those on placebo, as compared to 6.8% for those receiving EPA supplementation, a 20% relative reduction in recurrent stroke[17].

However, the mechanisms by which omega-3 PUFAs exert their plaque-stabilizing effects in carotid and other atherosclerotic plaques may be different to the cardioprotection observed with the omega-3 PUFA EPA. We demonstrate the association of increased inflammation and decreased omega-3 PUFA content in carotid plaques from neurologically symptomatic patients, and as such, it is plausible that omega-3 PUFAs may reduce inflammation in the atherosclerotic plaque, whereas EPA may be advantageous in the hyperlipidemic patient or patient status post-myocardial infarction via its anti-dysrhythmic mechanisms[3]. Recently, a large epidemiological study in Alaskan Eskimos found that high intake of omega-3 PUFAs does not protect against carotid atherosclerotic plaque formation but is associated with decreased intimal media thickness (IMT), an index of vascular aging and arteriosclerosis[18]; corroborating an earlier interventional study with EPA[19]. Whether omega-3 PUFAs help to prevent plaque rupture and instability through amelioration of an inflammatory cascade or through some other mechanism will be the subject of future research.

Recommendations have previously been made regarding the amount of omega-3 content which may prove to be beneficial for cardiac protection, especially in those at risk. The American Heart Association/American College of Cardiology recommends the intake of 1 g/day of EPA and DHA for the treatment post-myocardial infarction and prevention of sudden cardiac death [20]. Therapy with EPA and DHA can be monitored with the omega-3 index, a risk factor for sudden cardiac death[21].

Several limitations need to be pointed out about our pilot study. Since this is a pilot study, to test the hypothesis that carotid plaques from patients with neurological symptoms are inflammatory and have diminished omega-3 fatty acid content our sample size is rather modest (n=41). For example, using multivariable logistic regression, only diminished levels of DHA (OR 0.98, p=.024) and EPA (OR 0.97, p=.047) were associated with clinical symptoms, but the confidence levels are large enough such that a type I error cannot be excluded. However, we believe our findings lay the groundwork for future research into dietary and nutritional effects on carotid atherosclerotic plaque stabilization. Though there were no significant differences in over the counter fish oil supplements between the two groups (one in the asymptomatic group, none in the symptomatic group), dietary fish intake was not quantified between both groups. Hence, we are unable to determine if there is a dose-response relationship between dietary or nutritional fish oil supplementation and carotid atherosclerotic plaque vulnerability. If omega-3 PUFAs affect plaque stability, then they must then first be incorporated into the plaque. Thies et al previously demonstrated that this incorporation can indeed take place for omega-3 fatty acids [11]. The concentrations of EPA and DHA in plaques removed at CEA surgery were much higher in patients who consumed fish oil before surgery, compared to controls. Future research aimed at addressing this intriguing question is needed. Another limitation of our pilot study is that no serum inflammatory markers, such as high-sensitivity C-reactive protein and sedimentation rate were measured in this pilot study; this will be the subject of future research.

In the future, a study to address whether supplementation with dietary omega-3 PUFAs prevents carotid-related events in patients with moderate or high-grade carotid stenosis will help answer whether this is a formidable therapeutic target for the prevention of stroke. Future interventional trials will help to determine whether wide adaptation of EPA and DHA to patients with carotid atherosclerotic disease will lead to decreased carotid events.

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Figure 1.

Plaque stability correlates with inflammation. A) Asymptomatic carotid atherosclerotic plaques have an organized intima, compared to carotid plaques from neurologically symptomatic patients. Note the hemorrhage and intimal disruption seen in the symptomatic carotid plaque. (H&E staining, 40 $\times$ magnification; *: Denotes vessel intima). B) Immunohistochemistry demonstrates an increased content of CD68⁺ cells in the media of symptomatic carotid plaques compared to asymptomatic ones (open arrows). C) Symptomatic carotid atherosclerotic plaques have increased expression of CD68⁺ cells ($P = .003$). Mean with standard SEM; * P -value $\leq .05$ denotes significance; two-tailed unpaired t-test was used.

Figure 2.

Carotid plaques from neurologically symptomatic patients have diminished levels of omega-3, but not omega-6, fatty acids. A) Omega -3 fatty acid docosahexaenoic acid (DHA) is decreased in unstable plaques ($P = .019$) as is B) the omega-3 fatty acid eicosapentaenoic acid (EPA, $p=0.038$) but not C) the omega-6 fatty acid arachidonic acid (AA) ($P = .401$). Mean with standard errors of mean (SEM); * P -value $\leq .05$ denotes significance; two-tailed unpaired t-test was used.

Table 1

Patient demographics, comorbidities, and carotid atherosclerotic plaque classification.

	Carotid atherosclerotic plaques (n=41)		
	Asymptomatic (n=24)	Symptomatic (n=17)	P-value
<u>Demographics:</u>			
Age (years, mean ± SEM)	69.3 ± 2.7	64.8 ± 2.4	0.244
Male Sex	19 (83%)	16 (89%)	0.999
<u>Comorbidities:</u>			
Cardiac disease	6 (26%)	7 (39%)	0.265
Chronic renal insufficiency *	1 (4%)	1 (6%)	0.999
Diabetes mellitus	8 (35%)	6 (33%)	0.908
Hypertension	22 (96%)	15 (83%)	0.560
Smoker	10 (43%)	8 (44%)	0.833
<u>Medication use:</u>			
Aspirin	16 (70%)	16 (89%)	0.262
Clopidogrel	2 (9%)	5 (28%)	0.105
Oral hypoglycemic	7 (30%)	4 (22%)	0.264
Statin	18 (78%)	12 (67%)	0.999
<u>Plaque Characteristics:</u>			
Percent ICA Stenosis (mean ± SEM)	84.9 ± 3.6	81.1 ± 3.4	0.467
<u>Clinical history[†]:</u>			
Amaurosis fugax [‡]	-	2 (11%)	
Transient ischemic attack	-	9 (53%)	
Stroke	-	8 (44%)	

* Chronic renal insufficiency, serum Cr $\geq$ 1.6

[†] Ischemic symptoms occurring in the preceding six months

[‡] Temporary partial or complete loss of sight