Circulating inflammation-resolving lipid mediators RvD1 and DHA are decreased in patients with acutely symptomatic carotid disease

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ABSTRACT

Background: Efficient biomarkers for early prediction and diagnosis of an acutely symptomatic carotid plaque rupture event are currently lacking, impairing the ability to diagnose and treat patients with an acute plaque rupture events in a timely fashion. Resolvins are endogenous inflammation-resolving lipid mediators that are induced by inflammatory insults. We hypothesized that resolvin and other lipid profiles in sera likely mark the process towards plaque rupture.

Methods: Circulating lipids associated with plaque rupture events were quantitatively profiled via targeted mediator-lipidomics using ultra-performance liquid chromatography tandem mass spectrometry in patients with acutely symptomatic and asymptomatic carotid disease.

Results: Resolvin D1 (RvD1, 82 ± 11 pM vs. 152 ± 17 pM, p = 0.001) and docosahexaenoic acid (DHA) (0.052 ± 0.007 µM versus 0.076 ± 0.008 µM, p = 0.025) levels are decreased in the sera of patients presenting with an acutely symptomatic carotid plaque rupture event (n = 21) compared to patients with asymptomatic (n = 24) high-grade carotid stenosis. Circulating arachidonic acid (AA) levels, however, were higher (0.429 ± 0.046 µM versus 0.257 ± 0.035 µM, p < 0.01) in acutely symptomatic compared to asymptomatic carotid patients. ROC curve analysis demonstrates that the serum ratio AA:RvD1 (AUC 0.86, sensitivity 0.90, specificity 0.92) and AA:DHA (AUC 0.86, sensitivity 0.90, specificity 0.71) are biomarkers for the risk of atherosclerotic plaque rupture.

Conclusions: A circulating pro-inflammatory lipid profile, characterized by high AA:RvD1 and AA:DHA, is associated with acutely symptomatic carotid disease and stroke.

1. Introduction

Atherosclerotic plaques arise in the vessel intima and media and are thought to be a result of cholesterol deposition, hemodynamic strain, and inflammation [1]. Rupture of the fibrous cap leads to a transition from a stable to an unstable atherosclerotic plaque. This plaque rupture results in clinically relevant sequelae: in the coronary bed, an atherosclerotic plaque rupture leads to myocardial infarction and, in the carotid artery, to an ocular or cerebral ischemic event. These latter events manifest as amaurosis fugax, transient ischemic attacks, or stroke. There is a need for biomarkers that can be used by clinicians to diagnose the potential rupture of atherosclerotic plaques.

Gaps in knowledge remain in the mechanisms leading to acute symptomatic atherosclerotic plaque rupture. Increasing evidence suggests that unresolved inflammation contributes to the development of plaque rupture [2–4]. Endogenous omega-3 and omega-6 polyunsaturated fatty acids (ω3-PUFAs and ω6-PUFAs) and their metabolites likely play crucial roles in atherosclerotic rupture-associated chronic inflammation. Supplementation with ω3 docosahexaenoic acid (DHA)-rich fish oil results in fibrous cap thickening and stabilization [5]. We previously demonstrated that carotid plaques from neurologically symptomatic patients are inflammatory and have decreased intraplaque levels of ω3 fatty acids, including DHA [4]. Consistent with this observation, resolvin D1 (RvD1, 7S, 8R, 17S-trihydroxy-4Z, 9E, 11E,
1Z, 15E, 19Z-docosahexaenoic acid), a potent inflammation-resolving lipid mediator biosynthesized from DHA, was reported recently to promote plaque stability, including decreased lesion oxidative stress and necrosis, improved efferocytosis, and fibrous caps thickening [2]. Resolvins, including RvD1, are inflammation-resolving mediators initially uncovered in the resolution phase of inflammation. DHA-derived RvD1 contributes to the atherosclerotic plaque stabilization by DHA [2]. Arachidonic acid (AA) is the major ω-6 PUFA in humans and animals. AA is converted to a large array of short-lived pro-inflammatory eicosanoids including thromboxane A4, leukotriene B4, and sulfido-peptide leukotrienes and generally thought to promote inflammation. We aimed to determine whether circulating serum RvD1, DHA, and AA levels represent the risk of atherosclerotic plaque rupture.

2. Materials and methods

2.1. Study design

Patients with ≥ 50% internal carotid artery stenosis undergoing carotid endarterectomy (CEA) were included in this study. Informed consent was obtained after approval through the Ochsner Clinic Institutional Review Board. 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 medication use (anti-platelet, oral hypoglycemic, antihypertensive, or lipid-lowering). Any previous ipsilateral ischemic symptoms, including time from onset were also taken into account. Carotid plaque stability was determined on the basis of presenting symptomatology, confirmed by imaging (magnetic resonance imaging and/or computed tomography angiography imaging of the brain). Asymptomatic patients with high-grade carotid stenosis (≥ 80% internal carotid stenosis based on duplex ultrasound imaging) were included in the stable or asymptomatic carotid atherosclerotic plaque group. Patients presenting with symptoms of temporary or partial/complete loss of vision, a transient ischemic attack, and/or an established stroke with good neurologic recovery in the index hospitalization (7); mean time to intervention for acute interventions was 2.6 days (Table 1).

2.2. Lipid extraction and LC-MS/MS-based lipidomic analysis

The sera were isolated by centrifugation from the fasting peripheral blood of human patients who were identified as urgent/unstable symptomatic and asymptomatic carotid patients. The samples and associated information were de-identified after transfer from the Ochsner Clinic and handled according to the protocol approved by Institutional Review Board of Louisiana State University Health New Orleans. The extraction and LC-MS/MS-based lipid mediator analysis were performed following the protocols that others and we developed and used previously [4, 8-21] (http://www.hmdb.ca/metabolites/HMDB03733). Briefly, deuterium-labeled internal standards [2 ng of each, d4-prostaglandin D2 (d4-PGd2) and d5-DHA in 20 µl methanol] were added to each serum sample (100 µl each) at ~ 4 °C on water-ice to determine the extraction recoveries (typically > 80%) of the lipid mediators. Two volumes of ice-cold LC-MS/MS-grade methanol (EMD Millipore, MA) containing 0.005% butylated hydroxytoluene (BHT, to prevent auto-oxidation) and 0.1 mM acetic acid were added to each serum sample on ice. The mixtures were vortexed for 10 min. The supernatants were collected after centrifugation. The pellets were extracted two more times with 200 µl methanol:water (2:1) containing 0.005% BHT and 0.01 mM acetic acid. The extraction supernatants of each serum sample were pooled together and diluted with 10-volume water containing 0.005% BHT and 0.01 mM acetic acid. The mixture of each sample with apparent pH 4.5 was cleaned up with C18 solid phase extraction (500 µg/cartridge, Waters, Milford, MA); the cleaned extracts were reconstituted into 50% methanol and analyzed via LC-MS/MS for lipidomic analysis of eicosanoids and docosanoids.

The settings of the LC-MS/MS instrument were as follows. Xevo TQ-S triple quadrupole tandem mass spectrometry equipped with Acquity I Class UPLC (Waters) was used. The UPLC was carried out with an Acquity UPLC HSS T3 column (1.8-µm particle size × 2.1 inner diameter × 50 mm length). At 0.4 mL/min flowrate, the mobile phase ramped from 45% of solvent A (H2O + 0.01% acetic acid) and 55% of solvent B (methanol + 0.01% acetic acid) to 15% of solvent A and 85% of solvent B in 10 min, then ramped to 2% of solvent A and 98% of solvent B in 18 min, and then stayed at 2% of solvent A and 98% of solvent B until 25 min, and finally changed back to 45% of solvent A and 55% of solvent B and re-equilibrated until 30 min passed. The capillary voltage was – 2.5 kV. The desolvation temperature was 600 °C, and the desolvation gas flowed at 1100 L/h. The cone gas was 150 L/h, the nebulizer pressure was 7.0 bars, and ion source temperature was 150 °C. Argon collision gas was set at 0.13 mL/min and 3.8 mbar. The injection volume of the coupled autosampler was 10 µl.

LC-MS/MS data were analyzed using MassLynx version 4.1 software (Waters). The compounds in the serum samples were identified by matching the LC multiple reaction monitoring (MRM)-MS/MS ions and chromatographic retention times (within ± 0.1 min window) to those of the standards. The mixture of standards was analyzed between every 8 and 10 sample analyses at the same LC-MS/MS conditions as the sample analysis. The extraction recovery was determined using the deuterium-labeled internal standards. The quantities of compounds were calculated from areas of identified LC MRM-MS/MS chromatographic peaks, extraction recoveries, and calibration curves of compound standards. The concentrations (pico-molar, or pMs) of these lipids in sera (100 µl each) were then calculated. The identification of analytes was also confirmed by the full scan MS/MS spectra and LC chromatographic retention times acquired from samples via LC-full scan MS/MS after comparing with the standard compound.

Table 1

Patient demographics.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total (n = 45)</th>
<th>Urgent (n = 21)</th>
<th>Asymptomatic (n = 24)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, year</td>
<td>65.53 ± 8</td>
<td>63.57 ± 6.59</td>
<td>67.25 ± 9</td>
<td>0.14</td>
</tr>
<tr>
<td>Male sex</td>
<td>28</td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td>28.16 ± 5.08</td>
<td>28.13 ± 5.55</td>
<td>28.18 ± 4.75</td>
<td>0.97</td>
</tr>
<tr>
<td>Total cholesterol mg/dL.</td>
<td>173.79 ± 52.54</td>
<td>180 ± 58.91</td>
<td>167.82 ± 46.25</td>
<td>0.45</td>
</tr>
<tr>
<td>HDL mg/dL.</td>
<td>41.79 ± 16.24</td>
<td>42 ± 18.09</td>
<td>41.36 ± 14.69</td>
<td>0.86</td>
</tr>
<tr>
<td>LDL mg/dL.</td>
<td>96.21 ± 42.01</td>
<td>99.63 ± 46.66</td>
<td>92.79 ± 37.7</td>
<td>0.61</td>
</tr>
<tr>
<td>Triglycerides mg/dL.</td>
<td>184.98 ±</td>
<td>199 ± 146</td>
<td>171.32 ± 135.54</td>
<td>0.52</td>
</tr>
<tr>
<td>Serum creatinine mg/dL.</td>
<td>11.9 ± 0.5</td>
<td>1.3 ± 0.61</td>
<td>1.09 ± 0.37</td>
<td>0.18</td>
</tr>
<tr>
<td>Current (all) smoker</td>
<td>15 (33)</td>
<td>8 (17)</td>
<td>7 (16)</td>
<td></td>
</tr>
</tbody>
</table>

BMI, body mass index; HDL, High-density lipoprotein; LDL, low-density lipoprotein; all smoker refers to a history of prior but not active tobacco use.
Table 2
Sera concentrations of select inflammation-resolving lipid mediators and omega-3 (DHA) and omega-6 (AA) PUFAs quantified by LC-MS/MS.

<table>
<thead>
<tr>
<th>Lipids</th>
<th>Urgent</th>
<th>Asymptomatic</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA (C20:4-n6)</td>
<td>0.429 ± 0.046 (μM)</td>
<td>0.257 ± 0.035 (μM)</td>
<td>0.005</td>
</tr>
<tr>
<td>DHA (C22:6-n3)</td>
<td>0.052 ± 0.007 (μM)</td>
<td>0.076 ± 0.008 (μM)</td>
<td>0.025</td>
</tr>
<tr>
<td>EPA (C20:5-n3)</td>
<td>0.045 ± 0.004 (μM)</td>
<td>0.056 ± 0.004 (μM)</td>
<td>0.077</td>
</tr>
<tr>
<td>RvD1</td>
<td>82 ± 11 (pM)</td>
<td>152 ± 17 (pM)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Notes: Results are mean ± SEM, 21 acute patients and 24 asymptomatic patients; μM: micromolar/L serum; pM: picomolar/L serum.

RvD1, DHA, AA were purchased from Caymanchem.com (Cayman Chemical, Ann Arbor, Michigan) and used as external standards for the tuning, calibration, and other optimization of LC-MS/MS equipment. Deuterium-labeled d₄-prostaglandin D₂ (d₄-PGD2) and d₅-DHA from Cayman Chemical were used as the internal standards to determine the recoveries of lipid extraction and quantities of the analytics in LC-MS/MS analysis.

2.3. Statistical analysis

All data were presented in average ± standard error of the mean (SEM) and analyzed using Welch’s t-tests. A p-value of ≤ 0.05 was considered statistically significant. Logistic regression was used for multivariable analysis. SPSS software (www.ibm.com) was used for the statistical analysis. Logistic regression was used for ROC analysis. Clinical factors (gender, age) do not have a significant effect on each metabolite, with p values being 0.86 (gender) and 0.9 (age) in the DHA model, 0.92 (gender) and 0.2 (age) in the AA model, and 0.54 (gender) and 0.42 (age) in the RvD1 model. Thus, these factors were excluded from the logistic models. It is important to note that for the problem of possibly over fitting the logistic model for AUC, we found that AUC for the top predictors does not drop much after applying the leave-one-out cross validation procedure (AUC changes from 0.8611 to 0.8333 for AA:DHA, from 0.8353 to 0.7758 for AA:RvD1, and from 0.8988 to 0.8651 for the sum of the Z scores of DHA:AA and the Z scores of RvD1:AA). These indicate that there was no problem with severe over fitting. Modeling and calculation of related area under curve (AUC), sensitivity/specificity, positive likelihood ratio (LR+), negative LR (LR-), and accuracy were carried out in SAS and R software packages.

3. Results

3.1. Optimization of LC-MS/MS for lipidomic analysis of patients’ sera

There were no differences between the two patient groups’ demographics (Table 1). We extracted lipid mediators using methanol and solid phase extraction (SPE) technique as described previously [12]. SPE usually yields better recovery and cleanup of analysts than liquid-liquid extraction alone. We used LC MRM-MS/MS methodology to analyze the serum extracts because it offers high sensitivity, reproducibility, and selectivity to analyze small molecules, including the compounds targeted in this report [22–26]. Supplementary Table 1 and Supplementary Fig. 1 present the optimized MRM parent ions in Q1 sector, signature daughter ions in Q3 sector, cone voltages, collision voltages, and LC MRM-MS/MS retention times for each compound that, together, yielded the best sensitivity and reproducibility in analysis of inflammation-resolving resolving D series and lipoxin A₄, as well as their respective precursors DHA and AA. The optimization provided each compound a unique set of parameters that were different from those of other compounds (Supplementary Table 1 and Supplementary Fig. 1), which allowed for the specific detection of each compound by LC MRM-MS/MS. The identification of RvD1 in sera was verified further by an MS/MS full-scan spectrum acquired from a larger volume of sera of patients (Supplementary Fig. 2), which possessed diagnostic MS/MS ions of RvD1 as showed in the MS/MS spectrum of resolving D1 standard.

3.2. Resolvin D1 and ratio of arachidonic acid:resolvin D1 or arachidonic acid:docosahexaenoic acid; potential serum biomarkers for diagnosis of acutely symptomatic carotid plaques

Following the widely-used methodology to identify small molecules, including lipid mediators in the picogram range in physiopathological specimens [22–26], we used the criterion that the compounds identified in a patient’s sera must have their LC MRM-MS/MS chromatographic retention times (within ± 0.1 min window) and signature MRM product ions match to those of the authentic standards. RvD1 was identified by this criterion (Supplementary Fig. 1 and Supplementary Fig. 2). However, other resolvins and lipoxin A₄ were only detected when their amounts reached the minimal detection limits in one or two samples, thus they were not considered for biomarker potentials. DHA and AA were also identified in abundant quantities in sera (Supplementary Fig. 1).

Next, we quantified RvD1, AA, and DHA based on their LC MRM-MS/MS chromatographic peak areas, extraction recoveries, and linear calibration curves (Table 2). LC MRM-MS/MS chromatographic peak quantification of RvD1, AA, and DHA revealed that sera RvD1 concentrations were 82 ± 11 pM for acutely symptomatic (urgent) vs. 152 ± 17 pM for asymptomatic carotid patients (p = 0.001). Additionally, DHA (precursor of RvD1 biosynthesis) was lower in the sera of urgent, compared to asymptomatic carotid patients (0.052 ± 0.007 µM versus 0.076 ± 0.008 µM, p = 0.025). However, the serum concentration of AA, a major ω-6 PUFA, was higher in urgent vs. asymptomatic (0.429 ± 0.046 µM versus 0.257 ± 0.035 µM, p < 0.01); high levels occurred in stable plaques, while low levels occurred in the acute rupture. Note that AA and DHA are much more prominent in the sera as these are in the pM range, compared to RvD1, which is detected in the pM range.

Next, we investigated whether the sera concentration ratio of AA:DHA, AA:RvD1, and DHA:RvD1 could discern differences between the urgent and asymptomatic groups.

To investigate whether the concentration ratio of AA:DHA, AA:RvD1, DHA:RvD1, in the sera also could manifest differences between the acutely symptomatic and asymptomatic groups, we compared these ratios. The ratios of AA:DHA and AA:RvD1 were associated with urgent; both were higher in the sera of acutely symptomatic patients (10.3 ± 1.4 versus 4.1 ± 0.6, p < 0.01 and 7331.0 ± 1280.8 versus 2897.4 ± 932.0, p < 0.01, respectively), whereas there was no significant difference for DHA:RvD1 (Fig. 1A). Moreover, to determine whether the concentration ratio of AA:RvD1 or AA:DHA has an ability to predict the acute symptom of the carotid, the predictive values of these concentrations of AA, DHA, and RvD1 as well as their concentration ratios had AUC significantly greater than 50% (classification by chance). Both AUC and accuracy show that AA:DHA and AA:RvD1 perform best. Further comparisons show that the AUCs of AA:DHA and AA:RvD1 were not significantly different (p value = 0.72), but they were significantly different from that of DHA:RvD1 (p values = 0.0121 and 0.0002). The AUCs of DHA, AA, RvD1 were not significantly different (p values: 0.6147 for AA vs DHA; 0.4636 for DHA vs RvD1). More interestingly, we found that combining two concentration ratios improved the ability of predicting carotid plaque rupture. Using the sum of the Z scores of DHA:AA and the Z scores of RvD1:AA in ROC analysis,
the AUC increases to 0.90 (0.8069, 0.9907, 95% CI), which is significantly greater than 50%. This combination may provide us a powerful multi-parameter prediction tool.

4. Discussion

Unresolved chronic inflammation is a major factor in atherosclerotic plaque rupture. Resolvins are endogenous inflammation-resolving lipid mediators that are triggered by inflammation, likely as a self-defense action. In instances where resolvins are not adequately expressed, a pro-inflammatory state dominates [2,27–29]. Furthermore, in the case of atherosclerosis, inflammation is a component that is associated with the development of atherosclerotic plaque rupture [1,2]. Here we demonstrate that circulating resolvin levels correspond to carotid plaque stability. Utilizing a translational model of plaque rupture [30], serum levels of RvD1 and DHA, a precursor of RvD1, were found to be markedly lower in patients presenting with acute symptomatic plaque rupture events compared to patients with asymptomatic carotid disease. This correlates with our previous work demonstrating that acutely symptomatic plaques have a decreased content of anti-inflammatory lipids, such as DHA [4]. Additionally AA, the major ω6 PUFA in human blood, was detected with at higher ratios of AA/RvD1 or AA/DHA in the sera of acutely symptomatic carotid patients as compared to asymptomatic carotid patients. The mechanisms underpinning this observation deserves further study in the future.

As we searched for a biomarker of plaque rupture, we chose to focus on the concentration ratio of AA:RvD1 (pM:pM) in the sera because this incorporates the AA and RvD1 differences noted between the acutely symptomatic and asymptomatic carotid patients. This was confirmed by our observation that the AA:RvD1 ratio was significantly higher for acutely symptomatic patients than asymptomatic patients (Fig. 1A). The serum concentration ratio of AA:DHA had a similar trend. These ratios are unitless and dimensionless, which could reduce the systematic errors in the analysis of the lipids. Additionally, the ratio uses one value instead of two values, which could simplify the presentation and the discussion of the data. The ratio of AA:RvD1 is incorporated into routine clinical practice.

Efficient biomarkers for early prediction and diagnosis of acute symptomatic plaques are lacking, which impedes the ability of physicians to promptly diagnose and treat patients presenting, with an atherosclerotic plaque rupture event. Minimizing the time between plaque rupture and treatment is critical in reducing the morbidity after a patient has sustained a stroke as select patients diagnosed within 3 h are offered systemic thrombolysis with recombinant tissue plasminogen activator (tPA), which improves outcomes after stroke [31]. However, tPA utilization rates among acute ischemic stroke patients is less than 7%, mainly due to delay in diagnosis [7]. The novel RvD1-based biomarker candidates identified here aim to fill this gap. For example, a simple ELISA or other detection kits could be developed to quantify RvD1 in the sera and have the potential to minimize diagnostic delays, aid in patient selection for possible thrombolysis and intervention.

Furthermore, a serum biomarker predictive of plaque vulnerability and future stroke risk could also be helpful in the management of asymptomatic high-grade carotid stenosis. The stroke reduction benefit from CEA over medical therapy in patients with asymptomatic carotid disease is based on randomized controlled trials from the 1990s [32]. Since that time, however, both the safety of surgery (CEA) and medical management of carotid disease have improved [33]. Many new questions which is the best therapy for the patient with asymptomatic high-grade carotid disease [33]. Identification of circulating inflammation-resolving lipid mediators could, hypothetically, be used to predict which individuals with high-grade asymptomatic carotid disease are at risk of plaque rupture and stroke. Thus, earlier identification could then be used to treat such patients with more intense medical therapy or with a prophylactic CEA.

Although our experimental results provide a preliminary demonstration of the capability of RvD1 as a biomarker for the diagnosis and prediction of acute symptomatic plaque ruptures, we need to further confirm these results on a larger population of patients. Other precursors or metabolites of resolvin biosynthesis and degradation pathways also may be unique biomarkers for this purpose, and therefore this deserves further exploration. The profiles of whole fatty acids and enzyme systems warrant a thorough study in future research; this will help decipher the underlined mechanisms for our findings in this report. The LC-MS/MS based tools and protocols established in this report provide the basis for these future studies. These studies also will provide critical data that can facilitate the exploration of cellular and molecular mechanisms underlying RvD1 deficiency in the sera of acute symptomatic plaque patients. Future studies are needed to determine how these serum lipid profiles will aid in predicting carotid plaque vulnerability and how this could be incorporated into routine clinical practice.
Disclosures

None.

Summary

Fibrous cap rupture of a carotid atherosclerotic plaque leads to a transition from a stable to an unstable atherosclerotic plaque and is the etiology of a stroke. In a translational model for plaque rupture and utilizing ultrasensitive liquid chromatography tandem mass spectrometry, the sera of patients presenting acutely with a symptomatic carotid-related transient ischemic attack or stroke (n = 21) were found to have lower RvD1 and DHA levels compared to patients with asymptomatic carotid disease (n = 24). ROC curve analysis showed that the serum ratio AA:RvD1 and AA:DHA represent a circulating pro-inflammatory lipid profile that is associated with acutely symptomatic carotid disease and stroke. Future work will validate further if these could be biomarkers for the risk of atherosclerotic plaque rupture.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.plefa.2017.08.007.

References