ONLINE SUPPLEMENT

CYTOCHROME P450-DERIVED EICOSANOIDS AND VASCULAR DYSFUNCTION IN CORONARY ARTERY DISEASE PATIENTS

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SUPPLEMENTAL METHODS

Study Population
A cohort of 106 individuals with established and stable coronary artery disease (CAD), defined as ≥50% stenosis in one or more major epicardial coronary arteries by coronary angiography, were identified in the University of North Carolina (UNC) Cardiac Catheterization Laboratory between October 2007 and November 2010 [1, 2]. Exclusion criteria included pregnancy, atrial fibrillation, left-ventricular systolic dysfunction (ejection fraction ≤35%), current use of long-acting nitrates or insulin, active autoimmune disease, history of severe aortic stenosis, history of solid organ transplant or dialysis, or history of cancer within the previous 5 years. Eligible participants provided written informed consent and returned to the UNC Clinical and Translational Research Center 65±35 days after their index catheterization for a single morning study visit and blood sample collection after fasting overnight and withholding their morning medications. All study visits took place at least 7 days following the index catheterization, and all participants were clinically stable and chest pain free at the time of their study visit.

Participants were instructed to refrain from tobacco products, caffeine, and vigorous exercise the morning of the study visit, and from use of vitamin C, vitamin E, fish oil, niacin or arginine supplements, oral decongestants, non-steroidal anti-inflammatory drugs (other than low-dose aspirin), or phosphodiesterase-5 inhibitors for at least seven days prior to the study visit. Individuals who experienced a respiratory tract infection within four weeks of the study visit were not eligible to participate, but could be scheduled at a later date. The study protocol was approved by the UNC Biomedical Institutional Review Board and conducted in accordance with institutional guidelines.

Evaluation of Endothelial Function
Endothelial dysfunction is a physiologic manifestation of vascular inflammation secondary to impaired nitric oxide availability and function that is associated with future cardiovascular events in multiple patient populations [3]. Endothelium-dependent vasodilation was assessed by brachial artery FMD using a 12.5 MHz linear-array transducer (Philips HDI 5000 system) as previously described [1, 4]. All recordings were taken in the morning in a dimly lit room by the same sonographer. Following 10 minutes of rest, baseline brachial artery diameter was measured at end diastole from the lumen-intimal interface of the proximal and distal walls and calculated by averaging 10 consecutive frames. Reactive hyperemia was induced by inflating a blood pressure cuff around the right forearm for five minutes to a pressure of at least 70 mmHg greater than the systolic blood pressure. Upon cuff release, recordings were taken for 90 seconds and peak brachial artery diameter was assessed by averaging three consecutive frames. FMD was calculated as the peak percent change in brachial artery diameter from baseline [= 100*(diameter Peak – diameter Baseline) / (diameter Baseline)]. Following 10 minutes of rest, endothelium-independent vasodilation was quantified as the percent change in arterial diameter five minutes after sublingual administration of a 0.4 mg nitroglycerin tablet. All data were analyzed by Brachial Tools software (Medical Imaging Applications, Coralville, IA).

Quantification of Inflammatory Biomarkers
It is well-established that circulating biomarkers of inflammation are associated with prognosis in patients with established CAD [5]. Individuals with elevated high sensitivity C-reactive protein (hs-CRP, a systemic inflammatory mediator produced in the liver that correlates with cytokines including interleukin-6 [6]), cellular adhesion molecules (CAMs, inflammatory mediators expressed on endothelial cells that mediate leukocyte and platelet adhesion) and monocyte chemoattractant protein-1 (MCP-1, a chemokine synthesized in monocytes and
endothelial cells that drives monocyte recruitment to the vascular wall) levels, and genetic predisposition to higher epithelial neutrophil-activating protein (ENA)-78 levels (a chemokine synthesized in neutrophils and endothelial cells that drives neutrophil recruitment to the vascular wall) have each been associated with poorer survival in patients with established CAD [7-9].

Venous blood was collected from each study participant, and plasma and serum were separated by centrifugation. In fresh serum, hs-CRP was quantified by latex-enhanced turbidimetric immunoassay using the VITROS® 5600 Chemistry System (Ortho-Clinical Diagnostics, Inc., Rochester, NY) by UNC McLendon Clinical Laboratories. The remaining serum and plasma were aliquoted and stored at -80°C pending analysis. Plasma concentrations of CAMs (E-selectin and P-selectin) and the neutrophil (ENA-78) and monocyte (MCP-1) chemokines were quantified using the Human Adhesion Molecule and Human Cytokine Fluorokine® Multi-Analyte Profiling Kits (R&D Systems, Minneapolis, MN), respectively, with fluorescence detection on the Bio-Plex 200 System (Bio-Rad, Hercules, CA) according to the manufacturer's instructions.

**Quantification of Plasma Eicosanoids**

Plasma eicosanoids were quantified after solid phase extraction by high-performance liquid chromatography followed by tandem mass spectrometry (HPLC-MS/MS) as previously described [2, 10, 11]. HyperSep Retain PEP SPE cartridges (Thermo Scientific, Rockford, IL) were pre-conditioned with a solution of 0.1% acetic acid/5% methanol and spiked with 30 ng each of 10,11-epoxyheptadecanoic acid and 10,11-dihydroxynonadecanoic acid (internal standards). Plasma (0.25 mL) was diluted in 0.1% acetic acid/5% methanol containing 0.009 mmol/L butylated hydroxytoluene and added to the column. Samples were then washed with two volumes of 0.1% acetic acid/5% methanol, eluted in 1 mL of acetonitrile, dried under nitrogen gas at 37°C, and reconstituted in 40% ethanol.

Arachidonic acid derived metabolites from the CYP epoxygenase pathway (8,9-epoxyeicosatrienoic acid [EET], 11,12-EET, 14,15-EET, 5,6-dihydroxyeicosatrienoic acid [DHET], 8,9-DHET, 11,12-DHET, and 14,15-DHET) and CYP ω-hydroxylase pathway (20-hydroxyeicosatetraenoic acid [20-HETE]), and linoleic acid derived metabolites from the CYP epoxygenase pathway (12,13-epoxyoctadecenoic acid [EpOME] and 12,13-dihydroxyoctadecenoic acid [DHOME]) were separated by reverse phase HPLC on a 1x150 mm, 5μm Luna C18(2) column (Phenomenex, Torrance, CA) and quantified using a MDS Sciex API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) with negative mode electrospray ionization and multiple reaction monitoring. Data were captured and analyzed using Analyst 1.5.1 software. Relative response ratios of each analyte were used to calculate concentrations and extraction efficiency for each sample was calculated based on recovery of the internal standards. For samples in which the concentration fell below the lowest standard, a value of one half the lowest standard was imputed. Analytes for which more than 20% of the values were imputed were dropped from the statistical analysis [12]. Consistent with prior analyses [13], plasma 11,12-EET concentrations were below the lower limit of detection in 68 of 106 samples (64%), and therefore excluded from the analysis.

**Statistical Analysis**

Data are presented as mean ± standard deviation or median (interquartile range) unless otherwise indicated. CYP-derived eicosanoids and circulating biomarkers of inflammation did not follow the normal distribution and therefore were log-transformed prior to analysis. In order to account for batch variation in the quantification of inflammatory biomarkers, data within each batch were standardized using a z-score [i.e. (subject value – batch mean)/batch standard deviation], as described [14]. Correlations among each vascular function phenotype were determined by Pearson’s correlation. A significant correlation was observed between E-selectin and P-selectin (r=0.599, P<0.001). In order to minimize redundancy in our analysis, a
A consolidated ‘CAM score’ phenotype was calculated in each individual, as described [14], by summing the z-scores of E-selectin and P-selectin. No significant correlations were observed among the final five biomarkers of vascular function (Supplemental Table 1), indicating these biomarkers represent five distinct phenotypes.

In order to characterize the association between inter-individual variation in CYP-mediated eicosanoid metabolism and key vascular function phenotypes, plasma 20-HETE levels (the bioactive metabolite of the CYP ω-hydroxylase enzymes) were utilized as a biomarker of CYP ω-hydroxylase pathway function. The plasma 14,15-EET:DHET ratio (a sensitive in vivo biomarker of sEH metabolic function) [10, 15] and the sum of EET levels in plasma (the bioactive metabolites of CYP epoxygenase enzymes) were utilized as biomarkers of CYP epoxygenase pathway function as previously described (Supplemental Figure 1) [2].

For the primary analysis, associations between circulating biomarkers of CYP-mediated eicosanoid metabolism (20-HETE, 14,15-EET:DHET ratio, and sum EETs) and five distinct phenotypic indices of vascular function (FMD, CAM score, MCP-1, ENA-78, and hs-CRP) were evaluated by Pearson’s correlation. A secondary analysis was conducted using a model that adjusted for potential demographic (age, race, gender) and clinical (smoking status, diabetes, obesity, multivessel disease, hypertension, renin-angiotensin system inhibitor use, time post-catheterization [7-30 days, 31-90 days, >90 days]) confounders that associated with the biomarkers of CYP eicosanoid metabolism and/or phenotypic indices of vascular function in our population [2]. In order to account for the potential confounding effects of medications that may impact vascular function and/or CYP-mediated eicosanoid metabolism, each analysis was repeated after adding aspirin, clopidogrel, beta-blocker, or statin use to the adjusted model. We also conducted stratified analyses limited to subjects receiving aspirin (n=103), clopidogrel (n=83), beta-blockers (n=89), or statins (n=83).

In order to further assess the eicosanoid-vascular function relationships, vascular phenotypes were compared across eicosanoid metabolism tertiles by analysis of variance (ANOVA) and a post-hoc Student Newman-Keuls test. To minimize the impact of the multiple statistical tests required for this analysis, a false discovery rate (FDR) q-value was calculated for each comparison in our primary analysis, which is defined as the expected proportion of statistical tests deemed significant that are actually false-positives [16]. Only q-values for statistically significant findings (P<0.05) are presented. A post-hoc power analysis was completed to assess the statistical power of our study. All statistical analyses were performed using SAS Version 9.2 (SAS Institute, Cary, NC).
Supplemental Table 1. Correlation between vascular function phenotypes.

<table>
<thead>
<tr>
<th></th>
<th>FMD</th>
<th>CAM score</th>
<th>MCP-1</th>
<th>ENA-78</th>
<th>hs-CRP</th>
</tr>
</thead>
<tbody>
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<td>FMD</td>
<td>==</td>
<td>-0.064 (0.526)</td>
<td>-0.101 (0.313)</td>
<td>-0.013 (0.901)</td>
<td>0.002 (0.984)</td>
</tr>
<tr>
<td>CAM score</td>
<td></td>
<td>==</td>
<td>0.120 (0.225)</td>
<td>0.115 (0.244)</td>
<td>0.071 (0.474)</td>
</tr>
<tr>
<td>MCP-1</td>
<td></td>
<td></td>
<td>==</td>
<td>0.017 (0.863)</td>
<td>-0.093 (0.345)</td>
</tr>
<tr>
<td>ENA-78</td>
<td></td>
<td></td>
<td></td>
<td>==</td>
<td>0.094 (0.338)</td>
</tr>
<tr>
<td>hs-CRP</td>
<td></td>
<td></td>
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<td>==</td>
</tr>
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Data presented as Pearson’s correlation coefficient (p-value).
Supplemental Figure 1. Overview of CYP-mediated arachidonic acid metabolism. After release from the cell membrane by cytosolic phospholipase A\(_2\), free arachidonic acid is metabolized by CYP \(\omega\)-hydroxylases (CYP4A11 and CYP4F2) to 20-HETE or by CYP epoxygenases (CYP2J2, CYP2C8, and CYP2C9) to one of four EET regioisomers (5,6-, 8,9-, 11,12-, and 14,15-EET). The EETs (epoxides) are subsequently hydrolyzed by sEH to their corresponding DHET (diol) metabolites, which generally have less biological activity. Structures for the 14,15-EET regioisomer, the preferred EET substrate for sEH, and the 14,15-DHET metabolite are provided since the 14,15-EET:DHET (epoxide:diol) ratio is an established biomarker of sEH metabolic function.
REFERENCES