Resolvins D1, D2, and Other Mediators of Self-Limited Resolution of Inflammation in Human Blood following n-3 Fatty Acid Supplementation

Emilie Mas,1* Kevin D. Croft,1 Paul Zahra,1 Anne Barden,1† and Trevor A. Mori1†

BACKGROUND: Resolvins and protectins are families of local lipid mediators generated from the n-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) during self-limited resolution of inflammation. We aimed to develop a liquid chromatography–tandem mass spectrometry (LC-MS/MS) assay to measure these lipid mediators in human blood following n-3 fatty acid supplementation and to determine whether the blood collection method affects their measured concentration.

METHODS: Blood samples from 20 healthy volunteers enrolled in an n-3 fatty acid supplementation trial were collected in EDTA, heparin, or citrate, or prepared as serum after volunteers had undergone 3 weeks of supplementation. Plasma or serum was purified by solid-phase chromatography and analyzed with LC-MS/MS.

RESULTS: The assay identified 18R/S-hydroxy-5Z,8Z,11Z,14Z,16E-eicosapentaenoic acid (18R/S-HEPE); 17S-hydroxy-4Z,7Z,10Z,13Z,15E,19Z-docosahexaenoic acid (17R/S-HDHA); 7S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid (RvD1); 7S,8R,17R-trihydroxy-4Z,9E,11E,13Z,15E19Z-docosahexaenoic acid (17R-RvD1); 7S,16R,17S-trihydroxy-4Z,8E,10Z,12E,14E,19Z-docosahexaenoic acid (RvD2); 10S,17S-dihydroxy-4Z,7Z,11E,13Z,15E,19Z-docosahexaenoic acid (10S,17S-DiHDHA); and 10R,17S-dihydroxy-4Z,7Z,11E,13E,15E,19Z-docosahexaenoic acid (10R,17S-DiHDHA). The limits of detection and quantification were 3 pg and 6 pg on-column, respectively. The pathway precursors 18R/S-HEPE and 17R/S-HDHA, but not the resolvins, were lower in serum compared with plasma. After n-3 fatty acid supplementation, mean (SD) EDTA plasma concentrations were: 18R/S-HEPE 386 (56) pg/mL, 17R/S-HDHA 365 (65) pg/mL, RvD2 26 (4) pg/mL, RvD1 31 (5) pg/mL, and 17R-RvD 161 (7) pg/mL. 10S,17S-DiHDHA and PD1 concentrations were below the limit of quantification.

CONCLUSIONS: This is the first study reporting 17R/S-HDHA, RvD1, and RvD2 concentrations measured in human blood following oral n-3 fatty acid supplementation. The concentrations of the antiinflammatory lipid mediators RvD1 and RvD2 were within the biological range known to have antiinflammatory and proresolving activities in isolated human leukocytes and in vivo studies in mice.

© 2012 American Association for Clinical Chemistry

Inflammation is a major mechanism involved in many human diseases. Resolution of inflammation is an active process regulated by biochemical mediators and receptor-signaling pathways and driven by proresolving mediators. Serhan et al. (1) first described these potent antiinflammatory and proresolving lipid mediators derived from polyunsaturated fatty acids. They include lipoxins derived from arachidonic acid, E-series resolvins derived from the long-chain n-3 fatty acid eicosapentaenoic acid (EPA) (2) (Fig. 1) and D-series resolvins protectins/neuroprotectins and maresins, all derived from the n-3 fatty acid docosahexaenoic acid (DHA) (Fig. 2) (2–5). These mediators, acting via G-coupled protein receptors (6), have potent antiinflammatory and proresolving actions (7, 8) and reduce airway inflammation (9, 10), dernal inflammation (11), colitis (12), arthritis (13), and postoperative pain (14). Studies have shown that these mediators increase with time during the inflammatory process (1, 15).

1 School of Medicine and Pharmacology, Royal Perth Hospital Unit, University of Western Australia, Perth, Australia.
2 Address correspondence to this author at: School of Medicine and Pharmacology, University of Western Australia, Medical Research Foundation Bldg., GPO Box X2213, Perth, Western Australia 6847. Fax +61-8-9224-0246, e-mail emilie.mas@uwa.edu.au.
3 Anne Barden and Trevor A. Mori contributed equally to the work, and both should be considered as first authors.
Received May 22, 2012; accepted July 27, 2012. Previously published online at DOI: 10.1373/cchem.2012.190199
Two series of resolvins and protectins have been identified. One series includes those derived from EPA and DHA via lipoxygenase metabolism, referred to as the S-resolvins, S-protectins, and S-maresins (Figs. 1 and 2). The second series includes those derived from aspirin-triggered cyclooxygenase (COX-2) or cytochrome P450 metabolism of EPA and DHA. These lipid mediators are R-resolvins and R-protectins also known as aspirin-triggered resolvins/protectins (2, 3, 16, 17).

Fig. 1. Biosynthesis of RvE1 and RvE2 and aspirin-triggered RvE1 and RvE2 from EPA.

Fig. 2. Biosynthesis of D-series resolvins, protectins, and maresins, and aspirin-triggered D-series resolvins and protectins from DHA.
The aim of this study was to develop an assay that uses liquid chromatography–tandem mass spectrometry (LC-MS/MS) to simultaneously measure a number of lipid mediators of self-limited resolution of inflammation in human blood and to use this assay to measure their blood concentration following n-3 fatty acid supplementation. We also aimed to determine if the method of blood collection affects the measured concentration of lipid mediators.

Materials and Methods

18R/S-hydroxy-5Z,8Z,11Z,14Z,16E-eicosa-9,12-trans-15Z-19,20-trans-7,11,13,17,19-docosapentaenoic acid (18R/S-HEPE); 17S-hydroxy-4Z,7Z,10Z,13Z,15E,19Z-docosahexaenoic acid (17S-HEPE); 7S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid (RvD1) and Resolin (D1); 7S,8R,17R-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid (17R-RvD1 or 17R-Resolin D1); 7S,16R,17S-trihydroxy-4Z,7Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid (17S-RvD1); 10S,17S-dihydroxy-4Z,7Z,11E,13Z,15E,19Z-docosahexaenoic acid (10S,17S-dihydroxy-4Z,7Z,11E,13Z,15E,19Z-docosahexaenoic acid (10S,17S-dHDA)); and leukotriene B4-d4 (LTB4-d4) were purchased from Cayman Chemicals. Bond Elut C18, 500 mg, 3-mL cartridges were applied to solid-phase extraction cartridges (Bond Elut, C18 500 mg) and washed with 2 mL water and 2 mL methanol (50/50; vol/vol) for analysis by LC-MS/MS.

SAMPLE PREPARATION

Serum or plasma (1 mL) was thawed at room temperature and 500 pg LTB4-d4 added as the internal standard. After dilution with 2 mL of 100 mmol/L sodium acetate (pH 3) and acidification with acetic acid to pH 3, samples were applied to solid-phase extraction cartridges (Bond Elut C18 500 mg) and washed with 2 mL water and 2 mL hexane. Resolvins and protectins eluted with 2 mL ethyl acetate, dried under nitrogen and then reconstituted in 100 μL of 5 mmol/L ammonium acetate (pH 9)/methanol (50/50; vol/vol) for analysis by LC-MS/MS.

LC-MS/MS ANALYSIS

The LC-MS/MS analysis was performed on a Thermo Scientific TSQ Quantum Ultra triple-quadrupole LC-MS system equipped with an electrospray ionization source (ESI) operated in the negative ion mode. Instrument control and data acquisition were performed with Xcalibur 2.0.7 software. LC was performed on a Zorbax Eclipse XDB C18 column at ambient temperature. The mobile phases were (A) 5 mmol/L ammonium acetate pH 9, and (B) methanol at a flow rate of 400 μL/min. Chromatography used solvent A/B (50/50, vol/vol) from 0 to 1 min, then changed to A/B (30/70, vol/vol) from 1 to 15 min, and finally changed to A/B (50/50, vol/vol) from 18 to 21 min. For optimization of the mass spectrometer, standards 18R/S-HEPE, 17S-HDHA, RvD1, 17R-RvD1, RvD2, 10S,17S-diHDA, PD1, and LTB4-d4 (internal standard) at a concentration of 10 ng/μL were individually introduced into the mass spectrometer by direct infusion with a syringe pump at a flow rate of 10 μL/min into the HPLC solvent flow (flow rate 0.2 mL/min). The operating conditions for mass spectral analysis were as follows: spray voltage, 3500 V; capillary temperature and voltage, 350 °C and 35 V, respectively; sheath gas (nitrogen) and auxiliary gas pressure 60 and 50 psi, respectively. The mass spectrometer was employed in MS/MS mode with argon used as the collision gas (1.2 mTorr).

CALIBRATION CURVES

Quantitative analysis was performed using calibration curves of standards prepared in plasma rendered free of proresolving lipid mediators (stripped plasma) by passage through a solid-phase extraction column (Bond Elut, C18 500 mg) as described above. Linearity was determined by assaying increasing amounts of each lipid mediator in duplicate (0, 25, 50, 100, 200, 500, 1000 pg/mL) and a fixed amount of the internal standard LTB4-d4 (500 pg). The limit of detection was calculated using a signal-to-noise ratio of 3. The limit of quantification was determined using a signal-to-noise ratio of 10 as described by Masoodi et al. (18).

RECOVERY OF STANDARDS

Analyte recovery was determined by adding mixtures of the standards in the concentration range (50, 500, and 1000 pg/mL, n = 15 replicates) to stripped plasma.

INTRA- AND INTERASSAY VARIATION

Venous blood (15 mL) was collected into EDTA from 10 healthy volunteers and kept on ice until centrifugation (1500g at 4 °C). Pooled plasma was stripped as described above and 1-mL aliquots, stored at −80 °C, were used to determine the intra- and interassay variation of the assay. Intraassay variation was calculated from analysis in triplicate of plasma spiked with 3 concentrations (50, 500, and 1000 pg/mL) of 18R/S-HEPE; 17S-HDHA; RvD1; 17R-
RvD1; RvD2; 10S,17S-DiHDHA; and PD1. Interassay variation was calculated from triplicate analysis of plasma spiked with 3 concentrations (50, 500, and 1000 pg/mL) on 5 separate days. LTB4-d4 (500 pg) was added before solid-phase extraction.

**CONCENTRATIONS OF RESOVINS AND PROTECTINS IN HUMAN SERUM AND PLASMA**

Blood was obtained from healthy volunteers recruited from the general population who were already enrolled in an n-3 fatty acid supplementation trial. There were 15 men and 5 women, aged 50–67 years [mean (SD) 59(5) years]. In this population the mean (SD) BMI was 28(5) kg/m², systolic/diastolic blood pressure was 118 (14)/71(6) mmHg, fasting cholesterol was 5.0 (0.8) mmol/L, triglycerides 1.3 (0.5) mmol/L, and glucose 5.0 (0.3) mmol/L. Plasma and serum samples were collected after 3 weeks of taking 4 g fish oil/day (Blackmores Omega Daily®, 35% EPA and 25% DHA).

The participants gave informed written consent to participate in the study, which was approved by the human research ethics committee of the University of Western Australia. Fasting venous blood samples (15 mL) were prepared as serum (3.5 mL blood) or plasma from EDTA (5 mL blood), citrate (2.7 mL blood), or heparin (3 mL blood). Blood samples were collected on ice and centrifuged at 4 °C, and the supernatant (plasma or serum) was stored at 80 °C until analysis.

**STATISTICAL ANALYSIS**

SPSS v19.0 was used for statistical analysis. Values are presented as means and SDs or SEs. The linear response each standard curve in stripped plasma was assessed using the coefficient of determination ($R^2$).

**Results**

With LC-MS/MS we showed that individual standards of lipid mediators were well-resolved chromatographically (Fig. 3). MS/MS used collision energy optimized for each standard to generate the most abundant product ions and monitored ions specific for each lipid me-
mediator. The most prominent transition ions monitored in MS/MS for each lipid mediator (summarized in Fig. 3) were: RvD2, retention time (Rt) = 3.31 min, m/z 375.2 [M-1] (molecular ion − 1 mass unit), prominent product ions at m/z 174.9, 215, 259, 277, 295, and 357 with optimized collision energy 23, 18, 14, 18, and 14 eV, respectively; RvD1 and 17R-RvD1, Rt = 4.10 and 4.36 min, respectively, m/z 375.1 [M-1], prominent product ions at m/z 121, 135.1, 215.1, and 233.1 with optimized collision energy 31, 19, 18, and 15 eV, respectively; 10S,17S-DiHDHA and PD1, Rt = 7.58 and 7.95 min, respectively, m/z 359.2 [M-1], prominent product ions at m/z 137, 153, 188.2, and 206.2 with optimized collision energy 22, 17, 22, and 18 eV, respectively; LTB₄-d₄, Rt = 8.23 min, m/z 339.2 [M-1]; prominent product ion at m/z 197 with optimized collision energy 18 eV; 18R/S-HEPE, Rt = 10.50 min, m/z 317.2 [M-1]; prominent product ions at m/z 215.1, 255.1, 259.1, 273, and 299.2 with optimized collision energy 15, 15, 14, 13, and 13 eV, respectively; 17S-HDHA, Rt = 14.00 min, m/z 343.1 [M-1]; prominent product ions at m/z 201.1, 227.1, 245.1, 281.1, and 325.2 with optimized collision energy 14, 18, 13, 14, and 12 eV, respectively.

Calibration curves prepared from standard solutions showed the assay had excellent linearity up to 1000 pg (Fig. 4). The coefficient of determination (R²) for each was ≥0.97. The limit of detection was 3 pg on-column and the limit of quantification was 6 pg on-column. The mean recovery of each lipid mediator in stripped plasma spiked with 50, 500, or 1000 pg of standards was 73%, 96%, and 99%, respectively. The within-day precision of the assay was 1.3%–13.5% in samples analyzed in triplicate over a concentration range of 50–1000 pg/mL (Table 1). The between-day precision over the same concentration range performed in triplicate on 5 separate days ranged from 8.2% to 14.9% (Table 1).

RvD2, RvD1, 17R-RvD1, 18R/S-HEPE, and 17R/S-HDHA were measured in serum and plasma from healthy volunteers who had been enrolled in an n-3 fatty acid supplementation trial for 3 weeks (Table 2). The concentration of 18R/S-HEPE and 17R/S-HDHA was approximately 5–10-fold greater than that of their downstream pathway products. In plasma, both 18R/S-HEPE and 17R/S-HDHA were approximately 2-fold greater than in serum. Serum concentrations of RvD2, RvD1, and 7S,8R,17R-trihydoxy-4Z,9E,11E,13Z,15E19Z-docosahexaenoic acid (17R-RvD1) were comparable to those in plasma collected into different anticoagulants. There were no significant differences for any of the measured lipid mediators between plasma collected into the different anticoagulants. 10S,17S-DiHDHA and PD1 in plasma and serum were below the limit of quantification.

Fig. 4. Linear response of RvD2, RvD1, 17R-RvD1, 10S,17S-diHDHA, PD1, 18R/S-HEPE, and 17S-HDHA in stripped plasma (1 mL) spiked with increasing amounts (0, 25, 50, 100, 200, 500, 1000 pg) of each lipid mediator and a fixed concentration of the internal standard LTB₄-d₄ (500 pg).

Discussion

We describe for the first time an assay that simultaneously measures a number of EPA- and DHA-derived resolvins and protectins using LC-MS/MS in human samples following n-3 fatty acid supplementation. The assay has excellent reproducibility and precision. To
date the reports on resolvins and protectins have mainly focused on in vitro studies or small animal models of acute inflammation and there is a paucity of data on the concentrations of these lipid mediators in humans. We have shown that following n-3 fatty acid supplementation, the pathway precursors 18R/S-HEPE and 17R/S-HDHA, as well as the resolvins RvD2, RvD1, and 17R-RvD1, were present in concentrations that are known to have potent antiinflammatory and proresolving effects. Plasma concentrations of 18R/S-HEPE and 17R/S-HDHA, but not measured resolvins, were approximately 2-fold greater than in serum. We achieved optimization for the measurement of resolvins and protectins by LC-MS/MS. Using 5 different stripped plasma samples we determined that there were no interfering peaks present in the biological matrix at the retention times corresponding to any of the resolvins and protectins measured. The individual standards were well resolved chromatographically. In analyses we selected the most prominent product ion for quantification and a second ion to confirm identity. The assay had detection and quantification limits of 3 pg and 6 pg on-column, respectively, and showed linearity up to 1000 pg/mL with excellent recovery and precision.

In our assay the enantiomers RvD1 and 17R-RvD1 were separated chromatographically with retention times 4.10 and 4.36 min respectively. Given 18R- and 18S-series 5S,12R,18R-trihydroxy-6Z,8E,10E,14Z,16E-eicosapentaenoic acid (RvE1) and 5S,18R-dihydroxy-6E,8Z,11Z,14Z,16E-eicosapentaenoic acid (RvE2) are highly labile lipid mediators, we used racemic 18R/S-HEPE, which represents the precursor of these resolvins derived from Table 1. Within- and between-day assay variation of resolvins and protectins in human stripped plasma.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>RvD1</th>
<th>RvD2</th>
<th>17R-RvD1</th>
<th>105,17S-DihDHA</th>
<th>PD1</th>
<th>18R/S-HEPE</th>
<th>17S-HDHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within day</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 pg/mL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>48.2</td>
<td>54.2</td>
<td>40.4</td>
<td>60.4</td>
<td>33.4</td>
<td>50.9</td>
<td>32.7</td>
</tr>
<tr>
<td>SD</td>
<td>1.8</td>
<td>1.7</td>
<td>0.9</td>
<td>3.0</td>
<td>4.5</td>
<td>2.6</td>
<td>0.4</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>3.7</td>
<td>3.1</td>
<td>2.2</td>
<td>5.0</td>
<td>13.5</td>
<td>5.1</td>
<td>1.3</td>
</tr>
<tr>
<td>Mean</td>
<td>392.4</td>
<td>533.8</td>
<td>521.0</td>
<td>550.6</td>
<td>559.3</td>
<td>417.9</td>
<td>311.8</td>
</tr>
<tr>
<td>SD</td>
<td>14.8</td>
<td>22.0</td>
<td>37.3</td>
<td>13.9</td>
<td>14.9</td>
<td>13.9</td>
<td>28.5</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>3.8</td>
<td>4.1</td>
<td>7.2</td>
<td>2.5</td>
<td>2.7</td>
<td>3.3</td>
<td>9.2</td>
</tr>
<tr>
<td>500 pg/mL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>886.2</td>
<td>1256.2</td>
<td>938.2</td>
<td>1317.3</td>
<td>1360.0</td>
<td>813.2</td>
<td>558.7</td>
</tr>
<tr>
<td>SD</td>
<td>30.7</td>
<td>65.6</td>
<td>61.5</td>
<td>81.1</td>
<td>61.4</td>
<td>37.0</td>
<td>40.3</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>3.5</td>
<td>5.2</td>
<td>6.6</td>
<td>6.2</td>
<td>4.5</td>
<td>4.6</td>
<td>7.2</td>
</tr>
<tr>
<td>Between day</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 pg/mL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>34.6</td>
<td>34.0</td>
<td>28.8</td>
<td>42.1</td>
<td>26.7</td>
<td>50.6</td>
<td>29.9</td>
</tr>
<tr>
<td>SD</td>
<td>4.6</td>
<td>4.7</td>
<td>3.4</td>
<td>5.8</td>
<td>3.0</td>
<td>6.3</td>
<td>4.3</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>13.3</td>
<td>13.8</td>
<td>11.9</td>
<td>13.8</td>
<td>11.3</td>
<td>12.4</td>
<td>14.5</td>
</tr>
<tr>
<td>Mean</td>
<td>444.6</td>
<td>547.6</td>
<td>439.6</td>
<td>603.5</td>
<td>592.2</td>
<td>424.0</td>
<td>270.3</td>
</tr>
<tr>
<td>SD</td>
<td>64.9</td>
<td>58.5</td>
<td>66.5</td>
<td>61.1</td>
<td>55.4</td>
<td>42.9</td>
<td>40.4</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>14.6</td>
<td>10.7</td>
<td>15.1</td>
<td>10.1</td>
<td>9.4</td>
<td>10.1</td>
<td>14.9</td>
</tr>
<tr>
<td>500 pg/mL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>1042.5</td>
<td>1352.5</td>
<td>964.1</td>
<td>1281.0</td>
<td>1316.9</td>
<td>919.1</td>
<td>556.6</td>
</tr>
<tr>
<td>SD</td>
<td>155.3</td>
<td>152.9</td>
<td>98.8</td>
<td>108.9</td>
<td>108.5</td>
<td>94.3</td>
<td>66.1</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>14.9</td>
<td>11.3</td>
<td>10.2</td>
<td>8.5</td>
<td>8.2</td>
<td>10.3</td>
<td>11.9</td>
</tr>
</tbody>
</table>

* RSD, relative SD.
EPA metabolism via lipoxygenase, COX-2, or P450 (Fig. 1). Thus measurement of 18R/S-HEPE may represent total EPA-derived resolvin capacity. The enantiomers 18S-HEPE and 18R-HEPE can be resolved with baseline resolution by using chiral chromatography (19). Our analyses showed that 17S-HDHA and 17R-HDHA eluted as a single peak on LC/MS and had the same fragmentation pattern (data not shown). Although the 17S-HDHA was used to construct calibration curves and establish reproducibility, plasma and serum samples may contain both 17S-HDHA and 17R-HDHA. Measurement of 17R/S-HDHA most likely represents total DHA-derived resolvin capacity.

In healthy volunteers who took 4 g daily fish oil, which provided approximately 1.4 g/day EPA and 1.0 g/day DHA, for 3 weeks, we detected measurable concentrations of RvD2, RvD1, 17R-RvD1, 18R/S-HEPE, and 17R/S-HDHA in 1 mL of serum or plasma. The biological actions of RvD2, RvD1, and 17R-RvD1 have been extensively studied and each has been shown to have potent antiinflammatory and proresolving actions in vitro and in vivo small animal studies (1). 18R/S-HEPE is the precursor of 18R- and 18S-series RvE1 and RvE2. 18R-HEPE was first identified in inflammatory exudates from mice treated with n-3 fatty acids and aspirin (20). Oh et al. (19) also showed that both 18R-HEPE and 18S-HEPE were present in the serum of health volunteers 3 h after a single dose of 1 g EPA. Volunteers had previously taken 81 mg aspirin at 12 and 24 h before consuming EPA. We have shown that 3 weeks of n-3 fatty acid supplementation led to serum concentrations of 18R/S-HEPE of 191 pg/mL that compares favorably with the total 18R-HEPE and 18S-HEPE concentration (122 pg/mL) reported by Oh et al. (19). 17R/S-HDHA are the precursors of the 17R- and 17S-series of RvD1 and RvD2 (Fig. 2). To our knowledge this is the first report of the presence of 17R/S-HDHA, and RvD1 and RvD2 in human blood following oral supplementation with n-3 fatty acids.

Proresolving lipid mediators are locally acting autacoids whose concentrations would be expected to be highest at sites of inflammation where they exert their effects. An important finding of our study is that the concentrations of the potent antiinflammatory and proresolving agents RvD1 and RvD2 detected in blood from healthy humans after n-3 fatty acid supplementation for 3 weeks are within the bioactive range observed in isolated human leukocytes (21, 22) and in vivo studies in mice (12, 23).

Previous reports have used plasma or serum for measurement of resolvins and protectins and yet to our knowledge this important aspect has not been examined. We have shown that in human blood the concentration of 17R/S-HDHA was comparable to that of 18R/S-HEPE and both were present in quantities at least 5–10-fold greater than that of their downstream pathway products. The concentration of both 18R/S-HEPE and 17R/S-HDHA in plasma collected into different anticoagulants. The type of anticoagulant used for blood collection did not affect the concentration of the lipid mediators. The lower 18R/S-HEPE and 17R/S-HDHA in serum suggests that degradation of these lipid mediators may be occurring during the clotting process. Therefore, plasma concentrations may be more representative of circulating 18R/S-HEPE and 17R/S-HDHA.

Following n-3 fatty acid supplementation we observed peaks corresponding with 10S,17S-DiHDHA and PD1 in plasma and serum but these were below our criteria for the limit of quantification. It is possible that extraction of larger volumes

<table>
<thead>
<tr>
<th>Lipid mediator, pg/mL</th>
<th>Serum</th>
<th>EDTA</th>
<th>Heparin</th>
<th>Citrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>RvD1</td>
<td>24.4 (2.5)</td>
<td>31.4 (4.6)</td>
<td>33.0 (4.0)</td>
<td>40.6 (7.3)</td>
</tr>
<tr>
<td>RvD2</td>
<td>26.6 (4.7)</td>
<td>26.4 (3.6)</td>
<td>29.9 (3.8)</td>
<td>32.1 (4.9)</td>
</tr>
<tr>
<td>17R-RvD1</td>
<td>55.3 (6.0)</td>
<td>60.8 (7.3)</td>
<td>73.8 (7.4)</td>
<td>70.2 (4.5)</td>
</tr>
<tr>
<td>10S,17S DiHDHA</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>PD1</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>18R/S-HEPE</td>
<td>190.8 (16.6)</td>
<td>385.7 (52.6)</td>
<td>310.0 (22.8)</td>
<td>367.8 (28.0)</td>
</tr>
<tr>
<td>17R/S-HDHA</td>
<td>175.3 (32.2)</td>
<td>364.7 (65.0)</td>
<td>319.6 (64.5)</td>
<td>486.2 (227.3)</td>
</tr>
</tbody>
</table>

Values are given as mean (SE). LOQ = concentration below criteria for the limit of quantification (25 pg) in 1 mL of serum or plasma.

Table 2. Mean concentration (pg/mL) of lipid mediators in human blood prepared as serum or plasma collected in EDTA, heparin, or citrate following n-3 fatty acid supplementation.
of plasma or serum could enable their detection. However, to our knowledge 10S,17S-DiHDHA, and PD1 have not been reported in human blood. 10S,17S-DiHDHA has been measured in murine peritonitis but is produced only in trace quantities by human neutrophils (24). PD1 has potent immunoregulatory and neuroprotective actions (25) and is generated by isolated human leukocytes and tissues (24) and in particular by neural tissues where it is referred to as NPD1 (25). It is antifibrotic in the kidney and promotes wound-healing capacity (8).

The relevance of our findings to clinical medicine is highlighted by the range of conditions that may be influenced by RvD1 and RvD2. RvD1 has been shown to have antiinflammatory actions in animal models of inflammation. RvD1 provides protection against renal injury after ischemia reperfusion (26) and prevents neutrophil recruitment in peritonitis (27) and the dorsal skin air pouch (22). 17R-RvD1 is effective in preventing experimental colitis in the mouse (12). RvD2 is a potent regulator of leukocytes. It controls microbial skin air pouch neutrophil recruitment in peritonitis but is produced only in trace quantities. 10S,17S-DiHDHA has been measured in murine PD1 have not been reported in human blood. However, to our knowledge 10S,17S-DiHDHA, and PD1 have not been reported in human blood. 10S,17S-DiHDHA has been measured in murine peritonitis but is produced only in trace quantities by human neutrophils (24). PD1 has potent immunoregulatory and neuroprotective actions (25) and is generated by isolated human leukocytes and tissues (24) and in particular by neural tissues where it is referred to as NPD1 (25). It is antifibrotic in the kidney and promotes wound-healing capacity (8).

RvD1 and RvD2 have both been implicated as antinociceptive agents. RvD1 reduces inflammatory and postoperative pain in rodent models (29, 30), has been shown to inhibit transient receptor potential ankyrin 1 (TRPA1) in the dorsal root ganglion (31), and is an effective antihyperalgesic agent in a rat model of adjuvant-induced arthritis (13, 32). In mice, RvD2 is a potent inhibitor of TRPV1 (transient receptor potential subtype vanilloid) and TRPA1, 2 types of TRP channels that are strongly implicated in the genesis of inflammatory pain. RvD2 blocks synaptic plasticity that contributes to the development and maintenance of inflammation-induced pain (31). Therefore, the discovery of physiologically relevant levels of these mediators in human blood after n-3 fatty acid supplementation has the potential to broaden our understanding of a range of mechanisms involved in different clinical conditions.

In conclusion, we have described for the first time an assay that uses LC-MS/MS to measure a number of potent mediators of self-limited resolution of inflammation in human blood with excellent reproducibility and precision. We have shown that the EPA- and DHA-derived pathway precursors, as well as several resolvins, are present in plasma and serum following n-3 fatty acid supplementation. These findings highlight that biologically relevant concentrations of mediators of self-limited resolution of inflammation are achievable in healthy humans with n-3 fatty acid supplementation and thereby contribute to a better understanding of the mechanisms by which n-3 fatty acids exert their antiinflammatory action.

References