Impaired diurnal regulation of vascular RvD$_{n-3\ DPA}$ increases systemic inflammation and cardiovascular disease

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Abstract

Circadian mechanisms are central to regulating host responses. Recent studies uncovered a novel family of mediators termed as specialized pro-resolving mediators (SPM) that terminate inflammation without interfering with the immune response. Little is known on their circadian regulation. Using lipid mediator profiling and healthy volunteers herein, we found diurnal changes in n-3 docosapentaenoic acid-derived D-series resolvins ($RvD_{n-3 \ DPA}$) that peaked during the early morning hours. Lipid mediator profiling of plasma from patients at risk of myocardial infarct demonstrated reductions in $RvD_{n-3 \ DPA}$ that were associated with increased activation of peripheral blood platelets and leukocytes. Incubation of patient peripheral blood with $RvD2_{n-3 \ DPA}$ and $RvD5_{n-3 \ DPA}$ significantly reduced cellular activation. Furthermore, administration of $RvD5_{n-3 \ DPA}$ to Apolipoprotein E deficient mice significantly reduced platelet-leukocyte aggregates and vascular disease. These results demonstrate that peripheral blood SPM are diurnally regulated in humans and dysregulations in these pathways may lead to cardiovascular disease.

Introduction

Gaining an understanding of the basic mechanisms that regulate physiological responses to environmental changes is of interest since they may shed light into the ethiopathology of disease[1]. One basic environmental change that the body responds to is the light/dark cycle, which leads to a circadian regulation of a number of physiological functions, including leukocyte and platelet responses[2,3]. Disturbances to various aspects of these fundamental mechanisms are thought to be responsible for many of the diseases that afflict modern societies, including cardiovascular and metabolic disorders[2-4]. These conditions are characterized by a dysregulated inflammatory response, although the exact mechanisms that underlie this inflammatory state remain of interest.

Recent studies investigating the mechanisms engaged by the host to terminate ongoing inflammation uncovered a new genus of molecules, produced by leukocytes that reprogram both stromal and leukocyte responses[5-10]. These molecules, termed as specialized pro-resolving mediators (SPM), are produced *via* the enzymatic conversion of essential fatty acids, including n-3 docosapentaenoic acid (DPA), and are classified into four families: the lipoxins, resolvins, protectins and maresins[8]. The SPM actively counter-regulate the production of pro-inflammatory mediators, including cytokines and eicosanoids, and regulate leukocyte trafficking and phenotype following both sterile and infectious challenge[5-11]. In addition to having biological actions in peripheral organs, recent evidence indicates that these molecules may regulate processes occurring in the vasculature[10,12]. In this context the eicosapentaenoic acid-derived resolvin (Rv)E1 potently regulates platelet activation[11], maresin (MaR)1 and RvD2 protect against neointimal hyperplasia[12,13] and RvD1 promotes plaque stability in murine atherosclerosis[7]. Furthermore, plasma SPM concentrations were recently found to reflect outcome in sepsis[14] and increased plasma SPM in females are associated with

improved endothelial function following challenge when compared with males[15]. Thus, peripheral blood SPM concentrations may provide insights into both physiological and pathological processes ongoing in the vasculature.

Given the potent vascular actions of SPM and the correlation of circadian responses with a number of inflammatory conditions, including myocardial infarct[16,17], we investigated whether there were diurnal variations in systemic SPM levels. We also assessed whether the temporal regulation of SPM was associated with changes in leukocyte and platelet responses. Lipid mediator (LM) profiling of plasma from healthy volunteers demonstrated a significant increase in n-3 DPA-derived mediators, including the D-series resolvins ($RvD_{n-3\ DPA}$) between 7:00h and 9:00h. At these time intervals we also found increases in the expression of monocyte, platelet and neutrophil activation markers in healthy volunteer peripheral blood. Patients with cardiovascular disease demonstrated reduced plasma $RvD_{n-3\ DPA}$, a loss in diurnal regulation of these molecules and increases in the activation of circulating platelets, neutrophils and monocytes. Incubation of peripheral blood from these patients with $RvD2_{n-3\ DPA}$ and $RvD5_{n-3\ DPA}$ reduced the expression of specific platelet, monocyte and neutrophil activation markers. Furthermore, administration of $RvD5_{n-3\ DPA}$ to Apolipoprotein E deficient (Apo E)$^{-/-}$ mice reduced systemic leukocyte and platelet activation and protected from vascular disease.

Results

Diurnal changes in peripheral blood n-3 DPA-derived SPM are regulated by acetylcholine

To investigate whether peripheral blood SPM concentrations are diurnally regulated we obtained plasma from healthy volunteers at distinct intervals during a 24h period (see Supplementary Table 1 for demographics). LM were then extracted using C18 solid phase extraction and identified and quantified using liquid chromatography-tandem mass spectrometry (LC/MS-MS). In plasma from healthy volunteers we identified mediators from all four major essential fatty acid metabolomes, including the EPA derived E-series resolvins, n-3 DPA-derived resolvins and protectins, DHA-derived protectins and maresins and the arachidonic acid (AA)-derived prostaglandins and leukotrienes (Figure 1a). These mediators were identified in accordance with published criteria that include matching retention time in liquid chromatography and at least 6 diagnostic ions in the tandem mass spectrum[6] as illustrated for $RvD5_{n-3\,DPA}$ (Figure 1b). Multivariate analysis of plasma lipid mediator profiles demonstrated a diurnal shift in plasma LM-SPM concentrations with a leftward shift in LM-SPM clusters from morning to evening profiles (Figure 1c,d). This shift was associated with am increase in the amounts of n-3 DPA derived mediators, including $RvD1_{n-3\,DPA}$ and $RvD5_{n-3\,DPA}$ from the evening (18:00 h) to morning intervals (7:00 and 9:00 h) as well as increases in the inflammatory eicosanoids $PGF_{2\alpha}$ e Supplementary . We also found diurnal changes in plasma Thromboxane (Tx)B_2, the inactive further metabolite of the potent platelet agonist TxA_2[18] (Supplementary Table 2). Of note, concentrations of n-3 DPA derived SPM were within their reported bioactive[8,19] ranges suggesting that they may be involved in regulating vascular responses.

We next investigated the mechanism(s) by which peripheral blood n-3 DPA derived SPM may be regulated. Since we recently found that acetylcholine (ACh) regulates SPM production in leukocytes[20] we next assessed whether peripheral blood levels of this neurotransmitter were diurnally regulated. Here we found that plasma ACh concentrations mirrored those of the $RvD_{n-3\,DPA}$ reaching a maximum at 7:00 h (Supplementary Figure S2a), suggesting that ACh may also regulate the $RvD_{n-3\,DPA}$ in peripheral blood. To test this we incubated whole blood with ACh and investigated the n-3 DPA SPM concentrations using lipid mediator profiling. In these whole blood incubations we identified mediators from all four n-3 DPA mediator families in accordance with published criteria[6] (Supplementary Figure 2b-c; Supplementary Table 3). Quantitation of the identified molecules demonstrated increases in $RvD_{n-3\,DPA}$ (Supplementary Figure 2d; Supplementary Table 3) with a ~160 % increase in $RvD2_{n-3\,DPA}$ when compared with concentrations found in blood incubated with vehicle alone. Of note, incubation of peripheral blood with norepinephrine, another neurotransmitter that is diurnally regulated in the circulation[21] (n=7 volunteers), did not significantly augment the production of n-3 DPA derived mediators (n = 9 healthy volunteers; 0.1-10µM). These results suggest that ACh controls the diurnal changes in peripheral blood $RvD_{n-3\,DPA}$.

Circadian regulation of systemic leukocyte and platelet activation

Having found diurnal changes in peripheral blood LM-SPM levels and given the potent actions that $RvD_{n-3\,DPA}$ exert on leukocyte and platelet function[6,22] we next investigated whether this reflected changes in leukocyte and platelet activation. Flow cytometric analysis of peripheral blood cells demonstrated significant increases in neutrophil CD11b expression as well as an increase in platelet-neutrophil aggregates, measured as increases in the expression of CD41[23] on peripheral blood neutrophils (Figure 2a,b). We also found increases in CD11b and CD41 expression on circulating

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monocytes at the 9:00h interval compared with the 18:00h interval (Figure 2c,d). These results demonstrate a circadian regulation of leukocyte and platelet activation that reaches a maximum between 7:00 and 9:00h that is coincident with RvD$_{n-3\,DPA}$ concentrations.

RvD$_{n-3\,DPA}$ reduce leukocyte and platelet activation in peripheral blood

We next investigated the actions of RvD$_{n-3\,DPA}$ in regulating monocyte, neutrophil and platelet activation as well as platelet-leukocyte aggregates in light of their pathogenic functions in cardiovascular disease [24-26]. For this purpose we incubated human peripheral blood with platelet activating factor (PAF), given its role in propagating vascular inflammation[27], in the presence or absence of RvD$_{n-3\,DPA,}$. We then assessed the expression of activation markers on peripheral blood cells using flow cytometry. Incubation of human peripheral blood with RvD2$_{n-3\,DPA}$ led to dose dependent decreases in neutrophil CD11b expression and in the amounts of neutrophil-platelet aggregates measured as decreases in neutrophil CD62P (Figure 3a-d) and CD41 expression (n= 5 donors; ~20% decrease at 10 nM) when compared with cells incubated with PAF alone. In these incubations we also found a significant reduction in monocyte activation where RvD2$_{n-3\,DPA,}$ gave dose dependent decreases in monocyte expression of CD11b, and the platelet markers CD62P (Figure 3e,f) and CD41 (n= 5 donors; ~29% decreased at 10 nM). Similar findings were also made when healthy volunteer whole blood was incubated with RvD5$_{n-3\,DPA}$ that resulted in dose-dependent decreases in neutrophil and monocyte CD11b expression as well as in leukocyte-platelet aggregates (Figure 3). Of note in these incubations RvD1$_{n-3\,DPA}$ only partially regulate neutrophil, monocyte and platelet responses (n=5 donors) suggesting that each of the RvD$_{n-3\,DPA}$ displays specific biological actions in regulating vascular leukocyte and platelet responses. These findings also suggest that the observed increases in peripheral blood n-3 DPA SPM in the

morning hours (Figure 1, Supplementary Figure 1 and Supplementary Table 2) may form part of an endogenous protective program to counterregulate diurnal leukocyte and platelet activation.

Reduced RvD$_{n-3\ DPA}$ and increased systemic inflammation in peripheral blood from patients with cardiovascular disease

We next investigated whether results obtained with healthy volunteers were translatable to the clinical setting. Given that RvD$_{n-3\ DPA}$ increased during the early morning hours, a time window associated with higher incidence of myocardial infarct[28,29], we next investigated the peripheral blood levels of RvD$_{n-3\ DPA}$ in patients with cardiovascular diseases (CVD) that were also at an increased risk of myocardial infarct (see Supplemental Table 4 for details and methods for risk criteria). Using lipid mediator profiling we identified all three RvD$_{n-3\ DPA}$, including RvD5$_{n-3\ DPA}$ (Figure 4a,b) as well as mediators from the DHA, EPA and AA metabolomes including the D-series resolvins and the prostaglandins (Supplementary Table 5) in patient peripheral blood. Assessment of plasma RvD$_{n-3\ DPA}$ levels demonstrated significant decreases in both morning (9:00 h; am) and evening (16:00-18:00h; pm) concentrations in CVD patients when compared to the respective intervals in healthy volunteers (Figure 4c). In these patients we also found significant reductions in plasma concentrations of the RvD$_{n-3\ DPA}$ biosynthetic marker 7-HDPA[6] (Supplementary Figure 3). Furthermore, the ratio of plasma RvD$_{n-3\ DPA}$ to inflammation-initiating eicosanoids (prostaglandins, leukotriene B$_4$ and TxA$_2$) was significantly lower in these patients at both intervals measured when compared to healthy volunteers indicating an elevated systemic inflammatory status ($p < 0.05$). This was further supported by the observation that peripheral blood leukocyte and platelets also displayed an increased activation status. Flow cytometric analysis demonstrated increases in the expression of CD11b on both neutrophils and monocytes from CVD

patients when compared with healthy volunteers (Figure 4d, e). We also found increases

in platelet-neutrophil and platelet-monocyte aggregates in peripheral blood from CVD

patients when compared with peripheral blood from healthy volunteers (Figure 4f, g). Of

note, in peripheral blood from these patients we found a significant decrease in morning

plasma ACh concentrations compared to evening values (Figure 4h). Thus, these results

suggest that a failure to upregulate peripheral blood ACh leads to $RvD_{n-3 \, DPA}$ production

in patients with CVD.

Reduced leukocyte activation by $RvD2_{n-3 \, DPA}$ and $RvD5_{n-3 \, DPA}$ in patient peripheral blood

In order to test whether there was a relationship between the increased systemic

inflammation and reduced n-3 DPA derived SPM we next tested whether $RvD_{n-3 \, DPA}$

regulated patient peripheral blood leukocyte responses. For this purpose we incubated

whole blood from these patients with $RvD2_{n-3 \, DPA}$ and assessed cellular responses using

flow cytometry. $RvD2_{n-3 \, DPA}$ dose-dependently decreased platelet-neutrophil and platelet-

monocyte aggregates without significantly regulating CD11b expression (Figure 5).

Incubation of whole blood with $RvD5_{n-3 \, DPA}$ also led to a reduction in neutrophil-platelet

and monocyte-platelet aggregates with higher potency than $RvD2_{n-3 \, DPA}$ (Figure 5a,b). In

addition, $RvD5_{n-3 \, DPA}$ significantly reduced neutrophil and monocyte CD11b expression

(Figure 5c,d). We next tested whether the actions of these two mediators were also

retained in the presence of PAF[23,27]. Incubation of patient whole blood with either $RvD2_{n-3 \, DPA}$

or $RvD5_{n-3 \, DPA}$ led to decreases in platelet-neutrophil and platelet-monocyte

aggregates measured as decreases in CD62P (Supplementary Figure 4a,b) and CD41

expression (n=9 patients) on both leukocyte subsets. We also found that $RvD5_{n-3 \, DPA}$

decreased the expression of CD11b on neutrophils and monocytes, an action that was

only in part shared with $RvD2_{n-3 \, DPA}$ (Supplementary Figure 4c,d). These results suggest

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that reductions in circulating $RvD_{n-3 DPA}$ lead to increased circulating leukocyte and platelet activation in CVD patients.

$RvD5_{n-3 DPA}$ reduces systemic leukocyte and platelet activation and protects against vascular disease in Apo E$^{-/-}$ mice

We next investigated whether the protective actions of $RvD5_{n-3 DPA}$ observed with peripheral blood cells from both healthy volunteers and CVD patients were also retained *in vivo*. For this purpose Apo E$^{-/-}$ mice were fed western diet for 6 weeks and $RvD5_{n-3 DPA}$ (100ng/mouse; i.v.) was administered on alternative days for a two-week period. $RvD5_{n-3 DPA}$ administration reduced circulating platelet monocyte-aggregates, as measured by a decrease in both CD41 and CD62P expression on CD115 positive cells, and monocyte activation with a decrease in CD11b expression (Figure 6a). We also found a significant reduction in platelet-neutrophil aggregates and neutrophil activation with a >60% reduction in CD11b expression in mice given $RvD5_{n-3 DPA}$ when compared with mice given vehicle alone (Figure 6b).

Since platelet-leukocyte aggregates are involved in the pathogenesis of atherosclerosis[26] we next investigated whether $RvD5_{n-3 DPA}$ also protected against vascular disease. Oil red-O staining demonstrated a significant reduction in aortic lesions in mice given $RvD5_{n-3 DPA}$ when compared to mice given vehicle (Figure 6c). Furthermore, LC/MS-MS analysis of aortic sections demonstrated significant reductions in aortic prostanoids, with concentrations of TxB_2 being reduced by ~35% in mice given $RvD5_{n-3 DPA}$ (Figure 6d and Supplemental Table 6). Together these findings demonstrate that the protective actions of $RvD5_{n-3 DPA}$ on platelets and leukocytes are also retained *in vivo* leading to reduced vascular disease.

Discussion

In the present studies we uncovered a diurnal regulation of $RvD_{n-3\ DPA}$ in the vasculature of healthy volunteers. This upregulation in $RvD_{n-3\ DPA}$ coincided with an increase in platelet, monocyte and neutrophil activation during the morning hours. Circadian regulation of these pro-resolving mediators was controlled by the neurotransmitter ACh that was in turn also diurnally regulated in plasma of healthy volunteers. In CVD patients, we found significantly lower $RvD_{n-3\ DPA}$ compared with healthy volunteers. We also found a failure in the upregulation of these molecules during the early morning hours that was linked with a decrease in plasma ACh concentrations and increased peripheral blood leukocyte activation. Incubation of whole blood from both patients and healthy volunteers with $RvD2_{n-3\ DPA}$ or $RvD5_{n-3\ DPA}$ significantly reversed leukocyte and platelet activation. In addition, administration of $RvD5_{n-3\ DPA}$ to Apo $E^{-/-}$ mice using a therapeutic paradigm reduced systemic platelet and leukocyte activation and vascular disease. Together these findings indicate that disruption in the $ACh-RvD_{n-3\ DPA}$ axis may result in CVD.

It is now widely appreciated that physiological processes including cardiovascular function and the immune system are under the control of a molecular clock that oscillates following a diurnal pattern[2,3]. In the vasculature, platelet activation is at a maximum during the early hours of the day with the upregulation of several activation markers including CD62P[30]. Of note, this increase in platelet activation is coincident with an increase in plasma plasminogen activator inhibitor-1, a serine protease inhibitor that functions as the principal inhibitor of tissue plasminogen activator and urokinase, thereby increasing the risk of thrombosis[31]. Platelet CD62P mediates platelet-leukocyte interactions, a process that in addition to facilitating leukocyte recruitment to the vascular endothelium is also involved in leukocyte activation and the production of inflammatory mediators including cysteinyl leukotrienes[23], tumor necrosis factor-α and C-C motif

ligand-2[24,25]. CD62P enhances platelet adhesion to endothelial cells expressing

fratelkine, and triggers the release of Weibel-Palade-bodies in endothelial cells [24,25], thus

perpetuating the pro-inflammatory and pro-thrombotic status during the early hours of

the day. In addition, platelet-leukocyte aggregates are implicated in vascular disease

pathogenesis, including atherosclerosis[26]. Thus, these observations suggest that in

healthy individuals endogenous, diurnally regulated, protective mechanisms are

engaged that counterregulate this physiological inflammation to prevent vascular

inflammation and thrombus formation. In the present study, we found that plasma RvD_{n-3}

$_{DPA}$ concentrations increase during the early morning hours (Figure 1 and Supplementary

Table 2). These molecules were reduced in peripheral blood from patients at risk of

myocardial infarct that correlated with an increased leukocyte and platelet activation.

Furthermore, $RvD_{n-3\ DPA}$ regulated reduced leukocyte and platelet responses in peripheral

blood from both healthy volunteers and patients, and $RvD5_{n-3\ DPA}$ protected against

vascular disease in Apo $E^{-/-}$ mice (Figure 6). Thus, these findings indicate that alterations

in the diurnal regulation of these molecules may represent a key aspect in the

pathogenesis of cardiovascular diseases.

Pro-resolving mediators including $RvD2_{n-3\ DPA}$ and $RvD5_{n-3\ DPA}$ are produced *via*

the stereoselective conversion of essential fatty acids by enzymes that in the vasculature

are primarily expressed in leukocytes. The $RvD_{n-3\ DPA}$ biosynthetic pathway is initiated by

the conversion of n-3 DPA to 17S-hydroperoxy-docosapentaenoic acid, a step that is

catalyzed by the leukocyte 15-lipoxygenase (LOX). This is then converted to $RvD_{n-3\ DPA}$

by the leukocyte 5-LOX[6]. Of note, plasma concentrations of the $RvD_{n-3\ DPA}$ pathway

marker, and 5-LOX product[6], 7-HDPA were significantly reduced (Supplemental Figure

3). These finding are in line with published findings implicating the 5-LOX pathway as a

risk factor in developing cardiovascular disease[32].

Tissue pro-resolving mediator biosynthesis is also regulated by the vagus nerve *via* release of the neurotransmitter ACh, a mechanism that is central in maintaining tissue resolution tone[20]. Results from the present study demonstrate that the vascular levels of this neurotransmitter in healthy volunteers are diurnally regulated and increase during the early morning hours (Supplementary Figure 2), a mechanism that was dysregulated in patients with CVD (Figure 5). Thus, these results point to an uncoupling of plasma ACh regulation that leads to a reduction in the biosynthesis of $RvD_{n-3\ DPA}$ in CVD patients.

In summation, the present findings uncover a novel protective pathway that is centered on the diurnal regulation of vascular n-3 DPA-derived pro-resolving mediators. Increases in these molecules during the morning hours counterregulate physiological platelet and leukocyte activation limiting systemic inflammation and potentially vascular disease. In patients with cardiovascular disease, there is a significant loss in the production of these molecules with an increase in peripheral blood cell activation leading to increased systemic inflammation and CVD, including risk of myocardial infarct. In line with this notion, $RvD_{n-3\ DPA}$ reprogrammed circulating leukocyte and platelet activation, which in mice resulted in a significant reduction in vascular disease. Thereby, strategies to restore peripheral blood $RvD_{n-3\ DPA}$, including n-3 DPA supplementation that was recently shown to increase plasma $RvD5_{n-3\ DPA}$ in healthy volunteers[33], may be a useful therapeutic option. In addition, therapeutics based on the $RvD_{n-3\ DPA}$ may also provide new opportunities for fine-tuning the increased inflammatory status present in these patients, dampening systemic inflammation and reducing vascular disease.

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J.D., R.A.C and P.R.S designed and carried out experiments and analysed data; M.E.W carried out experiments and analyzed data; J.D. conceived overall research plan. All authors contributed to manuscript preparation.

Competing Financial Interests

The authors declare competing financial interests

Figure Legends

Figure 1. Vascular n-3 DPA-derived SPM are diurnally regulated in human healthy volunteers. Peripheral blood was collected from healthy volunteers at the indicated intervals and plasma placed in ice-cold methanol containing deuterium labeled internal standards. Lipid mediators (LM) were extracted, identified and quantified using LM profiling (see methods for details). (a) Representative MRM for identified LM, (b) MS-MS spectra used for the identification of RvD5$_{n-3\ DPA}$. Results are representative of n = 7 healthy volunteers. (c) PLS-DA 2-dimensional score plot of the distinct LM-SPM profiles identified human plasma at the indicated intervals and (d) corresponding 2-dimensional loading plot. Grey ellipse in the score plots denotes 95% confidence regions. Grey and blue circles represent LM with a variable in importance score ≥ 1; n=4 healthy volunteers per interval. (e) n-3 DPA concentrations identified and quantified at each of the time intervals. Results are mean ± s.e.m, n = 7 per time point and expressed as pg/mL. *, $p ≤$ 0.05 vs amounts at the 18h interval. Statistical analysis was conducted on the results in the white portion of the panel. Results in the grey panel are re-plotted from the white portion to aid in visualization of rhythmicity.

Figure 2: Diurnal regulation of peripheral blood leukocyte and platelet activation in healthy volunteers. Blood was collected at the indicated intervals and the expression of neutrophil, monocyte and platelet activation markers was assessed using fluorescently labeled antibodies and flow cytometry. (a,b) Neutrophil (a) CD11b and (b) CD41 expression. (c,d) Monocyte (c) CD11b and (d) CD41 expression. Results are mean ± s.e.m, n = 7 volunteers per interval and expressed as percentage of 18:00 h antigen expression. *p<0.05 vs 18:00 h interval, determined using repeated measures one-way ANOVA followed by Tukey's test.

Figure 3: RvD2 $_{n-3\ DPA}$ and RvD5$_{n-3\ DPA}$ reduce monocyte, neutrophil and platelet activation in healthy volunteer peripheral blood. Blood was collected from healthy volunteers and incubated with RvD2$_{n-3\ DPA}$, RvD5$_{n-3\ DPA}$ (0.1nM, 1nM or 10nM) or vehicle (PBS) for 15min (37°C) then with PAF (100ng/ml; 30 min; 37°C). Cell activation and leukocyte-platelet aggregates were assessed using flow cytometry. (a,b) Representative histograms depicting neutrophil (a) CD11b and (b) CD62P expression. (c,d) Cumulative neutrophil (c) CD11b and (d) CD62P expression. (e,f) Monocyte (e) CD11b and (f) CD62P expression. Results are mean of n = 5 per time point and expressed as percentage change from PAF incubated cells. *$p<0.05$ compared to PAF using one-sample t test, followed by Sidak correction of p values.

Figure 4. Systemic n-3 DPA-derived SPM are reduced and leukocyte activation is increased in patients with CVD. Peripheral blood from patients diagnosed with cardiovascular disease (CVD) was collected at 9:00 h (AM) and 16:00 - 18:00 h (PM). Plasma was placed in ice-cold methanol containing internal standards and LM identified and quantified using LM-profiling (see methods for details). (a) Representative MRM for the identified n-3 DPA SPM. (b) MS-MS spectra used for the identification of RvD5$_{n-3\ DPA}$. Results are representative of n = 9 CVD patients. (c) Plasma RvD$_{n-3\ DPA}$ concentrations. Results are mean ± s.e.m. and expressed as pg/mL. n = 9 CVD patients and n = 7 healthy volunteers (HV). *, $p \leq 0.05$ and **, $p \leq 0.01$ compared to indicated control. (d-g) Whole blood was incubated with fluorescently labeled antibodies and cell activation as well as leukocyte-platelet aggregates were assessed using flow cytometry. (d,e) CD11b expression on (d) neutrophils and (e) monocytes. (f,g) CD62P expression on (f) neutrophils and (g) monocytes. (h) Plasma ACh concentrations. Results are mean ±

s.e.m. and expressed as percentage antigen expression at 18:00 h interval. n = 5 HV and, 9 CVD patients. **p<0.01 compared to HV determined using Unpaired t-test.

Figure 5: RvD2$_{n-3 DPA}$ and RvD5$_{n-3 DPA}$ reduce leukocyte activation in peripheral blood from CVD patients. Peripheral blood from patients diagnosed with cardiovascular disease (CVD) was collected at 9:00 h. (a-d) Whole blood was incubated with RvD2$_{n-3 DPA}$ or RvD5$_{n-3 DPA}$ (0.1nM, 1nM or 10nM) or vehicle (PBS containing 0.01% EtOH) for 45min (37°C). Expression of CD62P on (a) neutrophils (b) monocytes and CD11b on (c) neutrophils and (d) monocytes was investigated using flow cytometry and fluorescently labeled antibodies. Results are mean ± s.e.m and expressed as percentage of Vehicle (Veh) incubated cells. n = 8 patients per interval. * p<0.05 *vs* Veh group using one-sample t test, followed by Sidak correction of p values.

Figure 6: RvD5$_{n-3 DPA}$ reduces systemic platelet and leukocyte activation as well as vascular disease in Apo E$^{-/-}$ mice. Apo E$^{-/-}$ mice were fed a western diet for 6 weeks and given RvD5$_{n-3 DPA}$ (100ng/mouse; i.v.) on alternate days for 2 weeks. Blood was obtained and (a) monocyte (b) neutrophil expression of CD41, CD62P and CD11b were determined using flow cytometry. (c) Aortic arches were stained using Oil red-O and staining intensity was determined using ImageJ. (d) Descending aortas were harvested and the levels of PGD$_2$, PGE$_2$, PGF$_{2\alpha}$ and TxB$_2$ quantified using LC/MS-MS. Results are mean ± s.e.m of 4-5 mice per group. * P <0.05 vs Vehicle treated mice using Students t-Test.

Methods

Materials. Liquid chromatography (LC)-grade solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA); Poroshell 120 EC-C18 column (100 mm x 4.6 mm x 2.7 µm) was obtained from Agilent (Cheshire, UK); C18 SPE columns were from Biotage (Uppsala, SE); synthetic standards for LC-tandem mass spectrometry (MS-MS) quantitation and deuterated (d) internal standards (d_5-RvD2, d_5-LXA$_4$, d_4-PGE$_2$, and d_4-LTB$_4$) were purchased from Cambridge Bioscience (Cambridge, UK) or provided by Charles N. Serhan (Harvard Medical School, Boston, Massachusetts, USA; supported by NIH-funded P01GM095467); Dulbecco's Phosphate-Buffered Saline (DPBS, without calcium and magnesium, Sigma), PAF C-16 (Cambridge Bioscience); Whole Blood Lysing Reagent Kit (Beckman Coulter, Inc); VioBlue-anti-CD41 (Miltenyi Biotec, clone REA386), PE-Cy5-anti-CD62P (BioLegend, clone AK4), Brilliant Violet 711-anti-CD11b (BioLegend, clone ICRF44), APC-Cy7-anti-CD16 (Abcam, clone 3G8), Alexa Fluor 647-anti-CD14 (BioLegend, clone HCD14). Anti-mouse CD11b-PE-Texas Red, CD62P-Brilliant Violet 650™, CD115-Brilliant Violet 711™, and CD41-Brilliant Violet 510™ (Biolegend).

Healthy volunteers blood collection: Venous Peripheral blood was collected at indicated intervals in sodium citrate (3.2%) from fasting volunteers that declared not taking NSAIDS for at least 14 days, caffeine and alcohol for at least 24h and fatty fish for 48h. Volunteers gave written consent in accordance with a Queen Mary Research Ethics Committee (QMREC 2014:61) and the Helsinki declaration. Blood was then taken for flow cytometry and lipid mediator profiling analysis.

CVD patients blood collection: Patients were screened and those that met the inclusion/ exclusion criteria were consented for blood to be obtained between 8:00 to 9:00 h and repeated between 16:00 to 18:00 h in accordance with East of England-Cambridge Central Research Ethics Committee and the Joint Research Management Office (JRMO), Queen Mary University of London. The inclusion criteria were i) severe coronary artery disease requiring treatment; ii) hospital admission for percutaneous coronary intervention (PCI); iii) >24hour post PCI; iv) able to provide informed consent; v) >18 years and vi) at least 2 of the following risk factors: hypertension, high cholesterol, smoker, diabetes, known ischemic heart disease

The exclusion criteria were: i) sustained ventricular tachycardia and/or ventricular fibrillation or appropriate ICD valve disease requiring intervention; ii) contra-indications to PCI; iii) women who are pregnant; iv) <18 years and v) enrolled in other studies. These blood samples were processed within 60 minutes of collection for *lipid mediator profiling* and *whole blood stimulations* as detailed in the sections below.

Targeted lipid mediator profiling. Plasma was obtained from peripheral blood of healthy volunteers and patients following centrifugation at 1500 x *g* for 10 min at room temperature. Descending aortas were weighed, placed in ice-cold methanol and homogenized using a glass dounce. All samples for LC-MS-MS-based profiling were extracted using solid-phase extraction columns as in[6,15]. Prior to sample extraction, deuterated internal standards, representing each region in the chromatographic analysis (500 pg each) were added to facilitate quantification in 4V of cold methanol. Samples were kept at -20°C for a minimum of 45 min to allow protein precipitation. Supernatants were subjected to solid phase extraction, methyl formate fraction collected, brought to dryness and suspended in phase (methanol/water, 1:1, vol/vol) for injection on a Shimadzu LC-20AD HPLC and a Shimadzu SIL-20AC autoinjector, paired with a QTrap

5500 or QTrap 6500 (Sciex). An Agilent Poroshell 120 EC-C18 column (100 mm x 4.6 mm x 2.7 µm) was kept at 50°C and mediators eluted using a mobile phase consisting of methanol-water-acetic acid of 20:80:0.01 (vol/vol/vol) that was ramped to 50:50:0.01 (vol/vol/vol) over 0.5 min and then to 80:20:0.01 (vol/vol/vol) from 2 min to 11 min, maintained till 14.5 min and then rapidly ramped to 98:2:0.01 (vol/vol/vol) for the next 0.1 min. This was subsequently maintained at 98:2:0.01 (vol/vol/vol) for 5.4 min, and the flow rate was maintained at 0.5 ml/min. QTrap 5500 was operated using a multiple reaction monitoring method as in (1). Each LM was identified using established criteria including matching retention time to synthetic and authentic materials and at least 6 diagnostic ions (1). Calibration curves were obtained for each using synthetic compound mixtures at 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100, and 200 pg that gave linear calibration curves with an r^2 values of 0.98–0.99.

Profiling of Acetylcholine and Norepinephrine. Plasma was placed in ice cold MeOH containing deuterated (d_9)-choline and kept at -20 °C for 45 min to allow for protein precipitation. Samples were then centrifuged for 10 minutes at 4000 x *g*. Supernatant were collected and evaporated under a gentle stream of nitrogen gas using a TurboVap LV (Biotage) at 37°C until dryness. Productes were then suspended in MeOH profiled using an LC/MS-MS system. A Qtrap 5500 (AB Sciex) equipped with a Shimadzu SIL-20AC autoinjector and LC-20AD binary pump (Shimadzu Corp.) was used with an Agilent Eclipse Plus C18 column (100x4.6mmx1.8µm). The mobile phase consisted of methanol/water/acetic acid, 80:20:0.01 (vol:vol:vol) for 2.5 min that was ramped to 98:2:0.01 (vol:vol:vol) over 0.2 min and maintained for 1.3 min. The flow rate was maintained at 0.5ml/min. To monitor and quantify the levels of acetylcholine and norepinephrine, the Qtrap 5500 was operated in positive mode and a multiple reaction monitoring (MRM) method was developed with signature ion fragments (m/z) for each

molecule monitoring the parent ion (Q1) and a daughter ion (Q3). The MRM transition employed for Acetylcholine was146>87 and for norepinephrine was 170>152.

Preparation of RvD1$_{n-3 DPA}$ and RvD2$_{n-3 DPA}$. RvD1$_{n-3 DPA}$ and RvD2$_{n-3 DPA}$ were prepared and isolated as in[6]. n-3 DPA (10 µM) was incubated with 100 U/ml isolated soybean-LOX (Borate buffer, 4°C, pH 9.2). 17S-HpDPA was isolated using UV-RP-HPLC (Infinity 1260; Agilent Technologies). 17S-HpDPA (10 µg) was then incubated with human neutrophils (80x10^6 cells/ml; PBS$^{+/+}$) and calcium ionophore (5 µM, 37°C). After 45 min the reaction was quenched using 2 volumes ice-cold methanol, reduced using sodium borohydrate, and products extracted using C18 SPE. RvD1$_{n-3 DPA}$ and RvD2 $_{n-3 DPA}$ were isolated using RP-HPLC (Infinity 1260; Agilent Technologies). Here, an Agilent Poroshell 120 EC-C18 column (100 mm x 4.6 mm x 2.7 µm) was kept at 50°C and LM isolated with a mobile phase consisting of methanol-water-acetic acid of 60:40:0.01 (vol/vol/vol) maintained for 2 minutes, then ramped to 80:20:0.01 (vol/vol/vol) from 2 min to 16 min and to 98:2:0.01 (vol/vol/vol) over 3 minutes. This was maintained for 2 min. Flow rate was kept at 0.5 mL/min.

Human whole blood incubations. In select experiments, whole blood was incubated with RvD1$_{n-3 DPA}$, RvD2$_{n-3 DPA}$, RvD5$_{n-3 DPA}$ (0.1, 1, 10 nM) or vehicle (PBS) for 15 min (37°C). Blood was then incubated with PAF (100nM) for 30 min (37°C). After stimulation, samples were washed twice with PBS for 12 min at 800 x g. Samples were stained for flow cytometry as described below.

In select experiments, venous blood from healthy volunteers was collected and incubated with acetylcholine (ACh) at 0.1 µM for 45 min (37°C). Plasma was then separated by centrifugation at 1,500 x g for 10 min for LM profiling.

Apo E$^{-/-}$ mice. Experiments strictly adhered to UK Home Office regulations (Guidance on the Operation of Animals, Scientific Procedures Act, 1986) and Laboratory Animal Science Association (LASA) Guidelines (Guiding Principles on Good Practice for Animal Welfare and Ethical Review Bodies, 3rd Edition, 2015). Apo E$^{-/-}$ mice were a kind gift of Prof. Fulvio D'Acquisto (Queen Mary Univesity of London). Mice (male and female) were fed a western diet for 6 weeks from 4 weeks of age and kept of a 12h light dark cycle. At 8 weeks of age mice were given RvD5$_{n-3\,DPA}$ (100ng/mouse; i.v.) or vehicle on alternate days for a 2-week period. Mice were culled, aortic arches were collected and stained using oil-red O as in[34]. Staining intensity was determined using ImageJ and expressed as relative units per mm^2. The descending aorta was collected, placed in ice-cold methanol and lipid mediators identified and quantified as described above.

Flow Cytometry. Whole blood was incubated with lineage-specific markers for 45 min (4°C, in DPBS containing 0.02% BSA). The following anti-human antibodies were used: VioBlue-anti-CD41, PE-Cy5-anti-CD62P, Brilliant Violet 711-anti-CD11b, APC-Cy7-anti-CD16, Alexa Fluor 647-anti-CD14. After staining, red blood cells were lysed using Whole Blood Lysing Reagent Kit, according to the manufacturer's instructions. Data was collected using BD LSR Fortessa and analysis was conducted using FlowJo (Tree Star Inc., V10).

In separate experiments blood was collected from Apo E$^{-/-}$ mice using heparin-lined syringes *via* cardiac puncture. Cells were incubated with Fc-blocking IgG and anti-mouse CD11b-PE-Texas Red, CD62P-Brilliant Violet 650™, CD115-Brilliant Violet 711™, and CD41-Brilliant Violet 510™ (Biolegend). for 45 minutes on ice. Red blood cells were lysed and fixed using Whole Blood Lysing Reagent Kit. Staining was then evaluated using LSRFortessa cell analyser (BD Biosciences) and analysed using FlowJo software (Tree Star Inc., V10).

Statistical analysis. Results are expressed as mean ± s.e.m. We assumed normality and equal distribution of variance between the different groups analyzed. Sample sizes for each experiment were determined on the variability observed in preliminary experiments. Differences between groups were assessed using one-sample t test (normalized data), Student's t test (2 groups), 1-way ANOVA (multiple groups) followed by post hoc Dunnett's test. Investigators were not blinded to group allocation or outcome assessment. The criterion for statistical significance was $p \leq 0.05$. Sample sizes for each experiment were determined on the variability observed in prior experiments[15] and preliminary experiments. Partial least squares-discrimination analysis (PLS-DA) and principal component analysis (PCA)[35] were performed using SIMCA 14.1 software (Umetrics, Umea, Sweden) following mean centering and unit variance scaling of LM levels. PLS-DA is based on a linear multivariate model that identifies variables that contribute to class separation of observations (Blister exudates) on the basis of their variables (LM levels). During classification, observations were projected onto their respective class model. The score plot illustrates the systematic clusters among the observations (closer plots presenting higher similarity in the data matrix). Loading plot interpretation identified the variables with the best discriminatory power (Variable Importance in Projection greater then 1) that were associated with Males (Blue) or Females (Red) and contributed to the tight clusters observed in the Score plot.

Figure 1

Figure 2

Figure 3

Figure 4

a Docosapentaenoic Acid-derived bioactive metabolome

b $RvD5_{n-3\ DPA}$

$361 = M-H$
$343 = M-H-H_2O$
$299 = M-H-CO_2-H_2O$
$281 = M-H-CO_2-2H_2O$

Figure 5