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Effect of omega-3 ethyl esters on the triglyceride-rich lipoprotein response to endotoxin challenge in healthy young men

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Abstract Oxylipins are produced enzymatically from polyunsaturated fatty acids, are abundant in triglyceride-rich lipoproteins (TGRLs), and mediate inflammatory processes. Inflammation elevates TGRL concentrations, but it is unknown if the fatty acid and oxylipin compositions change. In this study, we investigated the effect of prescription ω-3 acid ethyl esters (P-OM3; 3.4 g/d EPA + DHA) on the lipid response to an endotoxin challenge (lipopolysaccharide; 0.6 ng/kg body weight). Healthy young men (N = 17) were assigned 8–12 weeks of P-OM3 and olive oil control in a randomized order crossover study. Following each treatment period, subjects received endotoxin challenge, and the time-dependent TGRL composition was observed. Postchallenge, arachidonic acid was 16% [95% CI: 4%, 28%] lower than baseline at 8 h with control. P-OM3 increased TGRL ω-3 fatty acids (EPA 24% [15%, 34%]; DHA 14% [5%, 24%]). The timing of ω-6 oxylipin responses differed by class; arachidonic acid-derived alcohols peaked at 2 h, while linoleic acid-derived alcohols peaked at 4 h (p_{int} = 0.006). P-OM3 increased EPA alcohols by 161% [68%, 305%] and DHA epoxides by 178% [47%, 427%] at 4 h compared to control. In conclusion, this study shows that TGRL fatty acid and oxylipin composition changes following endotoxin challenge. P-OM3 alters the TGRL response to endotoxin challenge by increasing availability of ω-3 oxylipins for resolution of the inflammatory response.

Supplementary key words chylomicrons • fatty acids • inflammation • lipoprotein kinetics • omega-3 acid ethyl esters • VLDL • oxylipins • lipopolysaccharide • triglycerides • polyunsaturated fatty acids

Inflammation is a complex immune response to stimuli, such as infection, toxic exposure, stress, or injury, that includes the mobilization of immune cells, increased blood flow, and swelling (1). Systemic inflammation can occur when inflammatory signals are not cleared from the affected area or when bacterial endotoxin products, such as lipopolysaccharide (LPS), escape into the bloodstream (1, 2), which can lead to chronic low-grade inflammation if unresolved (2).

Oxylipins are enzymatically produced metabolites of PUFAs (**Fig. 1**) that are found abundantly esterified in lipoproteins such as VLDLs. The epoxide class of oxylipins produced from arachidonic acid (AA), epoxyeicosatrienoic acids (EpETrEs), have anti-inflammatory and antiatherogenic properties (3, 4). Epoxide products of the ω-3 PUFA, EPA, and DHA, epoxyeicosatetraenoic acids (EpETEs) and epoxydocosapentaenoic acids (EpDPEs), have similar, and possibly more potent, functions (5, 6). The EPA-derived alcohol, 18-hydroxyeicosapentaenoic acid (HEPE), decreases pro-inflammatory cytokine secretion from macrophages (7), and EPA- and DHA-derived alcohols are also important precursors for several pro-resolving lipid mediators (8). We have shown that rat livers treated with endotoxin have a reduced concentration of 17(18)-EpETE in VLDL and that treatment with an anti-inflammatory agent increases certain DHA epoxides (EpDPEs) (9), suggesting that the liver can regulate oxylipin signals transported in VLDL in response to endotoxin. However, regulation of oxylipins contained in human VLDL has not been demonstrated previously.

VLDL have inflammation regulating properties (10, 11), likely connected to oxidized fatty acids or oxylipins (12–16). The lipid content of VLDL can be made available to peripheral tissues through the lipolytic action of lipases such as lipoprotein lipase, and lipoprotein receptors such as the VLDL receptor, making oxylipin signals from VLDL available to the peripheral tissues (15). Previous studies have shown that lipoprotein lipase-mediated lipolysis of VLDL releases linoleic acid (LA)-derived oxylipins and induces endothelial inflammation (10, 11). We have shown that prescription

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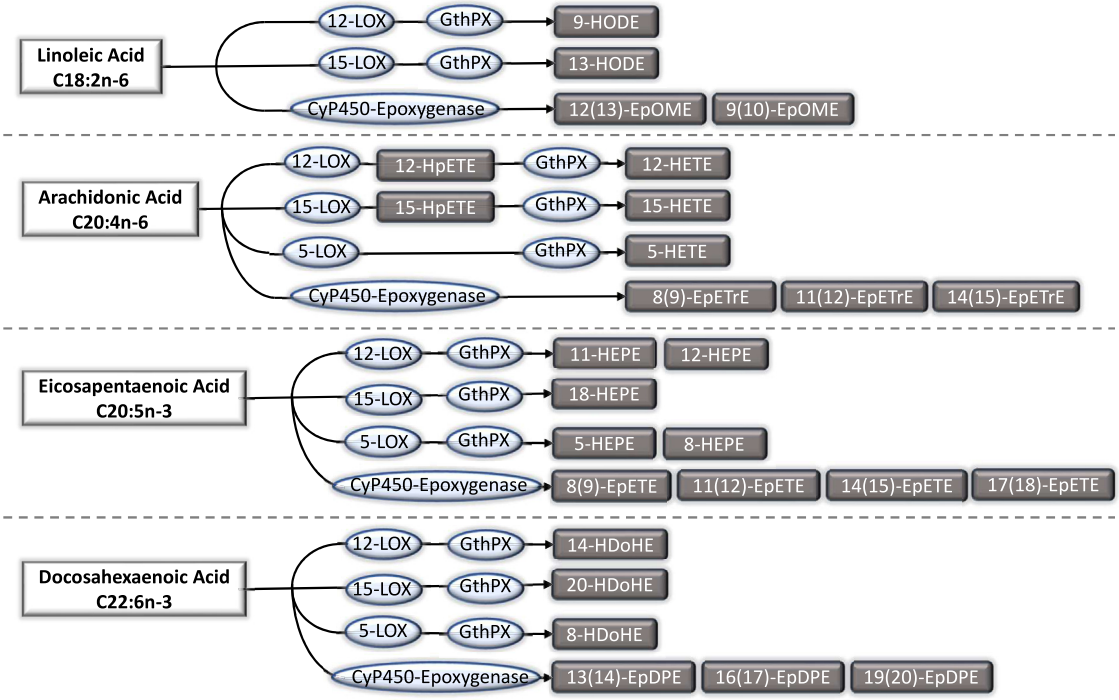

Fig. 1. Enzymatic production of oxylipins from parent polyunsaturated fatty acids. Enzymes (blue ovals) such as lipoxygenase (LOX), glutathione peroxidase (GthPX), and cytochrome P450 (CyP450) epoxygenase convert parent fatty acids (gray rectangles) into oxylipins (rounded rectangles). Oxylipins listed here are key molecules measured in this study. EpDPE, epoxydocosapentaenoate; EpETE, epoxyeicosatetraenoate; EpETrE, epoxyeicosatrienoate; EpOME, epoxyoctadecamonoenoate; HDoHE, hydroxydocosahexaenoate; HETE, hydroxyeicosatetraenoate; HEPE, hydroxyeicosapentaenoate; HODE, hydroxyoctadecadienoate; HpETE, hydroperoxyeicosatetraenoate.

ω-3 (P-OM3) treatment for 4 weeks alters lipoprotein oxylipin content by increasing ω-3 oxylipins and decreasing ω-6 alcohols, although ω-6 epoxides are largely unchanged (17). It is currently unknown how these differences in oxylipin concentrations may affect initiation and resolution of inflammation.

VLDL, produced in the liver, and chylomicrons, produced in the intestine, are together known as the triglyceride-rich lipoproteins (TGRLs) and are the main source of circulating triglycerides (TGs). Previous studies have documented the response of circulating lipids and lipoproteins to an inflammatory challenge in both animal and human models (18), observing elevated plasma TG and non-esterified fatty acids (NEFAs) in humans (19, 20) and rats (21, 22). In mice, peak plasma TG and NEFA concentrations occur 8 h after injection with endotoxin. In human subjects, endotoxin challenge results in peak VLDL and NEFA concentrations between 3 and 4 h (23, 24). In fasting animal models and cell models, endotoxin increases ApoB 100 (VLDL) and ApoB 48 (chylomicron) synthesis (25, 26), with a smaller increase in lipids (27). It is unknown if VLDL oxylipins follow a similar time course following endotoxin challenge.

Treatment with EPA and DHA is effective at reducing plasma TG and cholesterol concentrations in hyperlipidemic subjects (28, 29) and blunt the inflammatory response to endotoxin (30). We have previously shown their efficacy in insulin resistant subjects (31) is paired with an alteration in the TGRL oxylipin content (16). In humans, EPA + DHA doses less than 1 g/day do not change inflammatory response to endotoxin, and prescription doses of 3.4 g have not been shown to lower acute cytokine response to an endotoxin challenge in the first 24 h (32). However, plasma C-reactive protein measured over the course of a week is lower in ω-3–supplemented subjects (33). It is likely that the beneficial and anti-inflammatory effects of ω-3 fatty acids are due to changes in circulating fatty acid and oxylipin concentrations. We have previously observed that P-OM3 treatment increases nonesterified pro-resolving oxylipin concentrations following an endotoxin challenge in humans (33). However, TGRL oxylipin responses have not been previously characterized.

HYPOTHESES

In the current study, we investigated the response to acute inflammation by measuring the time-dependent response of fatty acids and oxylipins in the TGRL after a low-dose endotoxin challenge (34). We hypothesized that 1) an endotoxin challenge will induce time-dependent changes in TGRL fatty acid and oxylipin composition, 2) the response to an endotoxin challenge by the mid-chain alcohol class of TGRL-oxylipins will

be distinct from the anti-inflammatory epoxide class of oxylipin, and 3) treatment with long-chain P-OM3 will alter the oxylipin response to an endotoxin challenge by chemical class and parent fatty acid. Differential responses of specific fatty acids and oxylipins to the endotoxin challenge would suggest finely regulated control of individual fatty acids and oxylipins in lipoproteins, while differences in response with P-OM3 treatment would help to clarify how omega-3 fatty acids participate in this signal regulation.

MATERIALS AND METHODS

Participant selection and study design

This is an ancillary study from a crossover design trial of the effects of ω-3 treatment on inflammatory response to an endotoxin challenge (33). A cohort of healthy young men (N=20) were recruited and completed a baseline screening visit. Subjects were males (20–45 years old) who consumed less than two servings of oily fish per week. To be included, subjects were required to have a BMI of 20–30 kg/m^2, resting heart rate > 55 bpm, and fasting LDL-C < 160 mg/dl. Each subject was randomized to either olive oil control or P-OM3 acid ethyl ester treatment for 8–12 weeks. This treatment period was followed by a wash-out of approximately 8 weeks, after which the opposite treatment was administered for 8–12 weeks. P-OM3 treatment consisted of four capsules daily with 460 mg EPA-ethyl ester and 380 mg DHA-ethyl ester per capsule, for a total of 3.4 g/d of EPA + DHA (Pronova Biopharma; Oslo, Norway). Control treatment consisted of four daily olive oil capsules (4 g olive oil/day) that were free of both EPA and DHA (supplemental Table S1). Three subjects from the parent trial requested that their samples not be used for future research, so the sample size for the current study was 17. Informed consent was obtained from all subjects prior to participation. All study protocols were in accordance with Declaration of Helsinki principles and approved by the Penn State Institutional Review Board (NCT01813110).

Endotoxin challenge procedures

Following each 8–12 weeks treatment period, subjects reported to the clinical research center (CRC) at Penn State University Park campus for a low-dose endotoxin challenge. A sterile solution of protein-free endotoxin (US standard reference endotoxin; lot # CC-RE-LOT-1 + 2 + 3 from Clinical Center, National Institutes of Health) was prepared by reconstituting the lyophilized endotoxin powder in sterile water. The solution was mixed on a Metamix shaker for at least 30 min to ensure that no particles remained undissolved. Reconstituted vials were kept refrigerated at 2–8°C, and the solution was used within 24 h of mixing. The endotoxin challenge was administered as an intravenous injection of 0.6 ng LPS/kg body weight. Blood samples were collected at baseline and 1, 2, 4, and 8 h following LPS injection. Participants were instructed to fast for 12 h prior to testing and to avoid alcohol, strenuous exercise, and anti-inflammatory medications for 48 h prior to testing. Participants remained at the CRC for 8 h following endotoxin challenge for safety monitoring and IV saline was provided continuously for the duration of the test day.

Identical standardized meals, containing approximately 2400 kcal in total, were provided to participants on testing days. These meals consisted of prepared commercial foods that were representative of an average Western diet high in fat, refined carbohydrates, and sodium. Breakfast was consumed approximately 15–30 min following endotoxin administration and lunch was served after the 4 h blood sample. Study capsules were consumed with the standardized breakfast meal. Other foods included in the test meals contained no additional EPA or DHA.

Following collection of the 8 h blood sample, IV catheters and saline were discontinued and participants were briefly evaluated by a nurse to ensure stabilization of vital signs before being allowed to leave the CRC at approximately 8:00 pm. Participants were asked not to take any anti-inflammatory medications and were advised to notify the investigator team and limit any strenuous activity if they experienced symptoms. Subjects returned to the CRC for additional blood draws at 24, 48, 72, and 168 h. Participants were not required to fast or avoid alcohol or strenuous exercise in preparation for follow-up visits, but they were instructed to continue consuming the study capsules throughout the follow-up period. Because the uncontrolled nature of the follow-up period could complicate the lipid measurements in our study, we have excluded time points beyond the 8 h blood draw for this analysis. Blood was collected in EDTA tubes and centrifuged to remove erythrocytes from plasma. Plasma was stored at −80°C until analysis.

Clinical measurements

Plasma insulin, glucose, total cholesterol, HDL-C, and TG were measured at initial screening and at the baseline time point at each clinical visit by Quest Diagnostics. Fasting LDL-C was estimated using the Friedewald equation. Erythrocyte fatty acids were measured by gas chromatography, using standard methods previously described (OmegaQuant; Sioux Falls, SD) (35). The ω-3 index is reported as the sum of EPA and DHA as % of total erythrocyte fatty acids and was measured at screening and following each treatment period.

Time-dependent colorimetric analysis

Only baseline TG levels were measured by a clinical laboratory (Quest Diagnostics). Additional time-dependent samples were analyzed for TG response to endotoxin challenge by colorimetric assay. Plasma TG was measured for all time points by colorimetric absorbance assay using an Infinity enzyme kit (Thermo Fisher Scientific; Waltham, MA). Total TGRL protein in time-dependent samples was measured using the bicinchoninic acid method by colorimetric absorbance (Pierce MicroBCA Protein Assay, Thermo Fisher Scientific, Waltham, MA).

TGRL separation and ApoB analysis

TGRL fractions were isolated from EDTA plasma samples by density ultracentrifugation as described by Edelstein and Scanu (36). Plasma was diluted with a 1.0063 g/ml sodium chloride saline solution with BHT and EDTA as antioxidants. Enough solution was added to each plasma sample to fill a 4.7 ml Optiseal centrifuge tube (Beckman Coulter; Brea, CA). Samples were centrifuged at 45,000 RPM for 19 h at 4°C in a XL-70 Ultracentrifuge (Beckman Coulter). Following centrifugation, the top TGRL band was removed from the top of the tube and stored at -80°C until analysis.

A subset of four randomly selected subjects was analyzed for ApoB 100 and ApoB 48 in order to assess the lipoprotein synthesis response of the liver. TGRL fraction samples were prepared in a 2:1 ratio (sample:buffer) with 2× Laemmli Sample Buffer (Bio-Rad; Hercules, CA) with 2-mercaptoethanol and placed in boiling water for 5 min to denature disulfide linkages. Proteins were separated by 4–20% Criterion Tris-HCl, 26 well, precast polyacrylamide gel (Bio-Rad; Hercules, CA) with *Strep*-tagged recombinant 10-250kD protein ladder (Bio-Rad; Hercules, CA). Gels were run in pairs with Tris-glycine buffer at 100V and stained with SYPRO Orange Protein Gel Stain (5000 Concentrate in DMSO; Thermo Fisher Scientific, Waltham, MA) for 1 h. The gels were subsequently rinsed with 10% acetic acid and 10% glycerol in deionized water and immediately analyzed on Amersham ImageQuant 800 reader (Cytiva; Marlborough, MA) at UV (365 nm) and Cy2 (460 nm) fluorescence with 4x4 binning and quantified using ImageJ in browser (https://imagej.nih.gov/ij/). Area counts were used to calculate fraction of total protein, then concentrations were calculated using total TGRL protein measured by colorimetric absorbance assay.

TGRL oxylipin extraction and analysis

TGRL oxylipins were extracted and analyzed as previously described (17, 37). Briefly, total lipids were extracted from TGRL fraction samples using a modified Smedes protocol with a mix of deuterated oxylipin surrogates (9-HODE-d4, 9(10)-epoxyoctadecamonoenoic acid (EpOME)-d4, 12-HETE-d8, 14(15)-EpETrE-d11) for quantitation (38). Proteins were precipitated with isopropanol, and lipids were double extracted in cyclohexane. Samples were dried under nitrogen and reconstituted in 200 µl of 1:1 methanol:toluene. Lipid extracts were hydrolyzed by incubating for 1 h at 60°C in 0.5 M sodium methoxide in methanol and then adding water and incubating again for 1 h. Samples were diluted in 1 ml of a wash solution of 5% methanol and 0.1% acetic acid in water and loaded onto 3 ml Chromabond HLB solid phase extraction columns (Machery-Nagel; Duren, Germany) that had been preconditioned with the same wash solution. Columns were washed with 1 ml of a second solution of 25% methanol and 0.1% acetic acid in water, and then free oxylipins were eluted in 0.5 ml of 1.0% acetic acid in methanol first, followed by 0.5 ml of ethyl acetate. Finally, samples were dried under nitrogen and reconstituted in 1:1 methanol:acetonitrile that contained 100 nM 1-cyclohexyl ureido, 3-dodecanoic acid as an internal standard for quantitation.

Oxylipins were measured using a liquid chromatograph-mass spectrometer (Waters Xevo TQD, Acquity I-Class; Milford, MA) equipped with a CORTECS UPLC C18+ Column, 90 Å, 1.6 µm, 2.1 mm X 100 mm (Waters) for analyte separation. The column temperature was set to 40°C and a sample injection volume of 5 µl with a flow rate of 500 µl/min. Gradient elution was performed in a 15 min run. Solvent A was composed of water with 0.1% acetic acid, and solvent B was 90:10 (v:v) acetonitrile:isopropanol. An elution gradient of 25%–95% of solvent B was set from 0 to 11 min and held at 95% solvent B from 11 to 13 min. The system was then re-equilibrated and conditioned at 25% solvent B from 13 to 15 min.

Mass spectrometric analysis was performed using electrospray ionization in negative ion mode. Capillary voltage was 2.8 KV, source temperature 150°C, and desolvation flow of 1000 L/hr. Oxylipins were measured in multiple reaction monitoring mode using predetermined parameters by directly infusing individual oxylipin standards onto the MS to find the optimal mass transitions, cone voltage, and collision energy (supplemental Table S2). Detected oxylipins included alcohols and epoxides derived from LA, AA, EPA, and DHA. Calibration curves for each oxylipin were used to quantify concentration. Data were processed using TargetLynx Software (Waters; Milford, MA).

TGRL fatty acid analysis

To measure the fatty acid profile in TGRL, fatty acids from the TGRL fraction were methylated and analyzed by gas chromatography-mass spectrometry. A portion of the total lipid extract from the modified Smedes protocol described above was methylated by adding 200 µl of a 35:30:35 mix of 14% boron trifluoride in methanol:toluene:methanol and 200 µl of hexane with 10 µg/ml of methylated heptadecenoic acid (C17:1n9) as an internal standard. Samples were incubated at 100°C for 10 min and then allowed to cool before 200 µl of water was added. Samples were then centrifuged to extract methylated fatty acids in hexane. Fatty acid methyl esters in hexane were injected onto a QC2010 gas chromatography-mass spectrometry (Shimadzu; Kyoto, Japan) with a SP-2560 capillary column (Supelco; Bellefonte, PA). The mass spectrometer was run in scan mode with electron-impact ionization. Oven temperature started at 165°C and was slowly increased to 240°C to analyze fatty acid methyl esters with chain lengths from 12 to 22 carbons.

Statistical methods

To examine changes over time, mixed models with repeated measures were used. Time and treatment were fixed variables, while visit and subject were included as random variables. The spatial power covariance structure was used to allow covariance between samples of adjacent time points. In time-dependent tests, multiple comparisons were adjusted using the false discovery rate correction term, q, with an acceptable false discovery rate value of 10% (q = 0.1) (39). When comparing screening measures with baseline measures after treatment, Tukey HSD post hoc analysis was used. All statistical analyses were performed using JMP Pro 15.0 (SAS Institute, Inc.; Cary, NC).

RESULTS

Participant characteristics at screening are presented in Table 1. Subjects were generally healthy, young, and normal weight, with normal baseline values of plasma glucose, insulin, TG, and cholesterol. Subjects were 65% White, 29% Asian, and 6% Black.

TABLE 1. Participant characteristics at screening (All were male; N = 17)

Characteristics (Units)	Mean (±SD)
Age (yrs)	26 (5)
BMI (kg/m^2)	25 (2)
Systolic blood pressure (mg/Hg)	118 (10)
Diastolic blood pressure (mg/Hg)	77 (6)
Plasma glucose (mM)	5.1 (0.4)
Plasma insulin (mM)	4.5 (0.5)
Total cholesterol (mg/dl)	164 (25)
HDL-C (mg/dl)	52 (11)
LDL-C (mg/dl)	96 (18)
Plasma triglycerides (mg/dl)	81 (39)

P-OM3 treatment increased ω-3 index from 4.4% (% of total erythrocyte fatty acids) at screening to 8.8% after 8–12 weeks of treatment (**Fig. 2**A). There was no significant change with control, indicating a sufficient washout period in the crossover design and no carry-over effect. P-OM3 treatment did not have any significant effect on plasma TG, HDL-C, LDL-C, or glucose. However, participation in the study resulted in lower plasma insulin, regardless of treatment (Fig. 2B, C). Plasma TG peaked at 18% [95% CI: 5%, 33%] over baseline at 4 h in the postendotoxin and postprandial period after the standardized breakfast meal, but there was no effect of treatment group on plasma TGs (**Fig. 3**). The TG, ApoB 48, and ApoB 100 response varied widely between individuals (supplemental Fig. S1).

Fatty acid fractional abundance over time differed by fatty acid (**Fig. 4**), supporting our first hypothesis that specific fatty acid concentrations in TGRL are regulated at the level of individual fatty acids in response to endotoxin challenge. Fractional LA and dihomo gamma linoleic acid did not change over time, but on control treatment, fractional AA content was 16% lower [95% CI: 4%, 28%] at 8 h following endotoxin challenge in the control group. As expected, P-OM3 treatment increased EPA and DHA enrichment in TGRL at baseline and across all time points. On P-OM3 treatment, fractional EPA increased from baseline by 24% [95% CI: 15%, 33%] at 4 h following endotoxin challenge, and fractional DHA increased from baseline by 14% [95% CI: 5%, 24%] at 2 h following endotoxin challenge. Hence, ω-3 fatty acids were a component of the early TGRL response following acute inflammation, and P-OM3 treatment improved their availability.

TGRL oxylipin response to endotoxin challenge followed a pattern similar to plasma TG, with a peak at 4 h (**Fig. 5**A), broadly supporting our first hypothesis

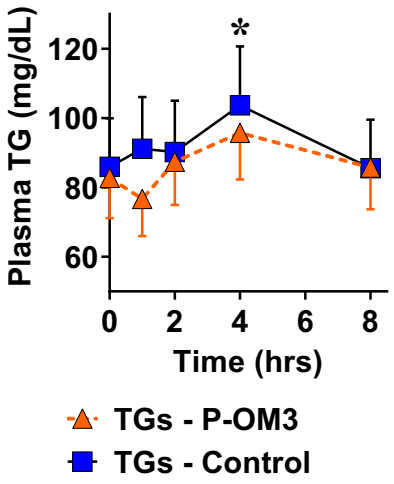

Fig. 3. Time-dependent triglyceride response to endotoxin challenge. Plasma triglycerides (TGs) increased after endotoxin challenge (n = 17), with a peak concentration at 4 h. Blue squares represent control treatment; orange triangles represent P-OM3 treatment. Differences from baseline were assessed by repeated measures ANOVA with mixed models. Post hoc analysis was done using the False Discovery Rate Correction term, q, with the acceptable false discovery rate of 10%, *$P < 0.05$, different from baseline adjusted for false discovery rate, q = 0.1 (39). Data is graphed as least-squares means ±95% confidence interval. P-OM3, prescription ω-3 acid ethyl ester.

that there would be a regulated time-dependent response of the TGRL oxylipins. After 4 h, oxylipin concentrations decreased slightly by 8 h. This same pattern was observed in both alcohols and epoxides. We expected that the alcohol response to endotoxin challenge would be more pronounced. Mean alcohols increased by 63% [95% CI: 43%, 85%] at 4 h (Fig. 5B), while mean epoxides increased by 47% [95% CI: 25%, 74%] (Fig. 5C). Alcohols were generally more prevalent than epoxides, but the fractional increase from baseline

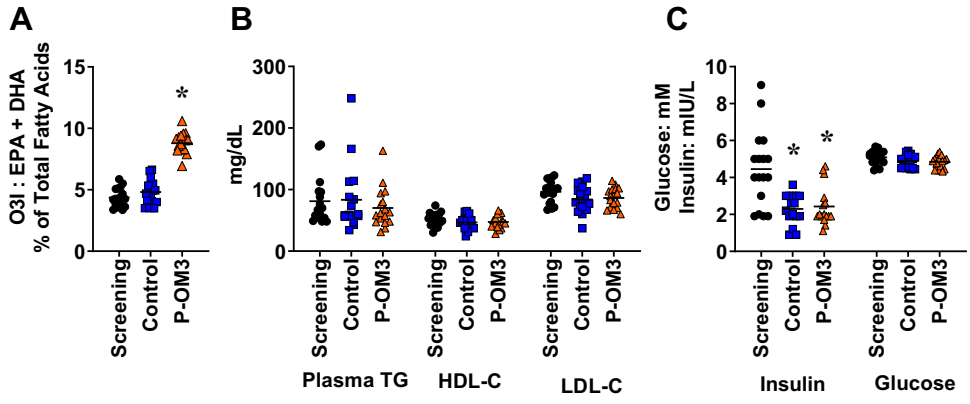

Fig. 2. Effect of prescription ω-3 ethyl ester treatment on blood lipids, glucose, and insulin. A: P-OM3 treatment increased ω-3 index (O3I) by 99% (95% CI: 87%, 111%), while olive oil control resulted in no change. Differences were assessed by repeated measures ANOVA with mixed models, and post hoc analysis was done by Tukey HSD post hoc test, *$P < 0.05$. B: P-OM3 and control had no significant effect on baseline plasma triglycerides (TGs), HDL-C, or LDL-C. C: Both control and P-OM3 had an unexpected effect of decreasing plasma insulin concentration, while there was no effect on plasma glucose concentration. Differences were assessed by repeated measures ANOVA with mixed models, and post hoc analysis was done by Tukey HSD post hoc test, *$P < 0.05$. P-OM3, prescription ω-3 acid ethyl ester.

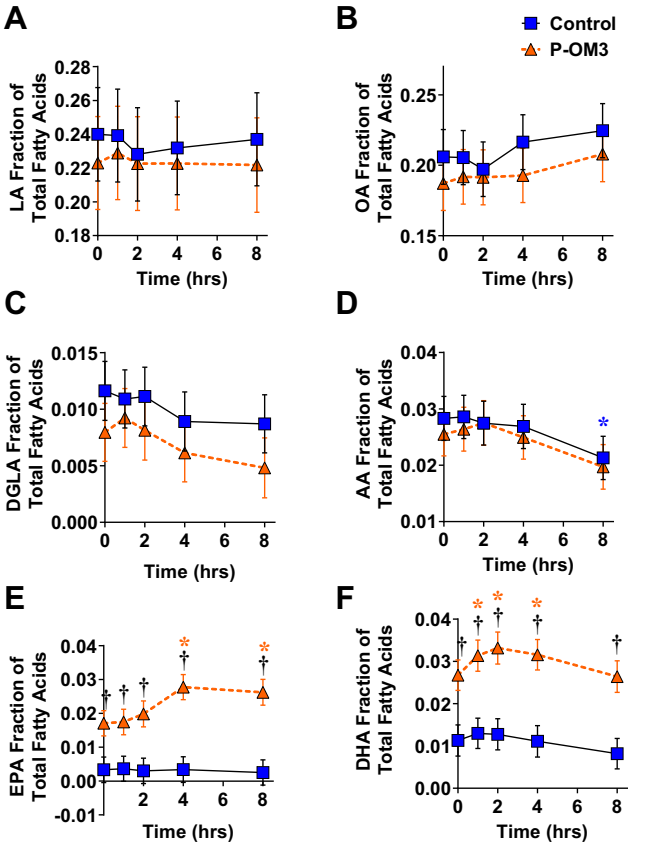

Fig. 4. Effect of endotoxin on TGRL fatty acid composition. A: TGRL fractional linoleic acid (LA; C18:2n6) did not change over time after endotoxin challenge in either control or P-OM3 treatment groups. B: TGRL fractional oleic acid (OA; C18:1n9) did not change significantly over time after endotoxin challenge in either control or P-OM3 treatment groups. C: TGRL fractional gamma linolenic acid (DGLA; C20:3n6) did not change significantly over time after endotoxin challenge in either control or P-OM3 treatment groups. D: Fractional arachidonic acid (AA; C20:4n6) composition was lower at 8 h in control group. E: Fractional TGRL eicosapentaenoic acid (EPA; C20:5n3) was increased at both 4 and 8 h with P-OM3 treatment. P-OM3 treatment increased overall EPA enrichment by 432% (340%, 523%; $P < 0.0001$) across all time points. F: Fractional TGRL docosahexaenoic acid (DHA; C22:6n3) was increased at 1 and 2 h with P-OM3 treatment. P-OM3 treatment increased overall TGRL DHA enrichment by 142% (120%, 164%; $P < 0.0001$) across all time points. Blue squares represent control treatment; orange triangles represent P-OM3 treatment. Differences were assessed by repeated measures ANOVA with mixed models. Post hoc analysis was done using the False Discovery Rate Correction term, q, with the acceptable false discovery rate of 10% (39). Fatty acid abundance expressed as fraction of total mass. N = 15; samples from two subjects were unavailable for fatty acid analysis. Longitudinal data is graphed as least-squares means ±95% confidence interval. * $P < 0.05$ adjusted for false discovery rate, q = 0.1; Different from baseline time point. † $P < 0.05$ adjusted for false discovery rate, q = 0.1; P-OM3 different from control. P-OM3, prescription ω-3 acid ethyl ester; TGRL, triglyceride-rich lipoprotein.

was not statistically different between classes, which does not clearly support our second hypothesis that alcohols would have a different response than epoxides.

In **Fig. 6**, we further tested our second hypothesis at the level of individual parent fatty acid and chemical class. We found that most oxylipin classes (HODEs, HEPEs, hydroxydocosahexaenoic acids, EpOMEs, and EpDPEs) reached peak concentration at 4 h after endotoxin challenge, similar to total oxylipins and plasma TG (Fig. 6A). However, differences in the timing of AA oxylipins were evident: AA alcohols (HETEs) reached peak concentration at 2 h (Fig. 6B), and AA epoxides (EpETrEs) reached peak concentration at 8 h (Fig. 6F). The time-dependent response of HETEs was significantly different from that of HODEs (p_{int} = 0.006; Fig. 6J), demonstrating that TGRLs were first enriched with HETEs, followed by HODEs during acute inflammation. Both EPA alcohols (HEPEs; Fig. 6C) and DHA epoxides (EpDPEs; Fig. 6H) were higher with P-OM3 than with control at 4 h (HEPEs, 161% [95% CI: 68%, 305%]; EpDPEs, 178% [95% CI: 47%, 427%]). EpDPEs were also higher with P-OM3 at 2 h (113% [95% CI: 12%, 306%]). AA-derived hydroperoxides (hydroperoxyeicosatetraenoic acid, HpETEs) are biochemical precursors of HETEs. HpETEs appeared to peak at 4 h then gradually decreased, but there were no significant differences from baseline in either treatment (Fig. 6I). These results support our first and second hypotheses by suggesting that different TGRL oxylipins, specifically AA-derived alcohols and epoxides, responded differently to endotoxin challenge, since the fractional oxylipin profile changed from baseline to peak oxylipin response at 4 h (Fig. 6J). In addition, our third hypothesis was supported because the response of EPA-derived alcohols and DHA-derived epoxides was dependent on P-OM3 treatment status.

Similar results are seen in the response of individual oxylipins. The LA-derived oxylipins 9-HODE, 13-HODE, 9(10)-EpOME, and 12(13)-EpOME responded very similarly to each other (**Fig. 7**A–D). However, the response of some other oxylipins differed by regioisomers within the class. 12-HpETE was elevated at 2 h with control, but 4 and 8 h with P-OM3 (Fig. 7E). However, the 15-HpETE response was similar to the response of total HpETEs, with an apparent peak at 4 h followed by a gradual decrease, but no changes from baseline reached statistical significance (Fig. 7F). When HETEs were separated into individual regioisomers, only 5-HETE had a significant change from baseline peaking at 2 h, but both 12-HETE and 15-HETE also appeared to peak at 2 h, similar to total HETEs (Fig. 7G–I). The response of HEPEs and EpDPEs were similar to their respective total oxylipin classes with significant peaks at 4 h with P-OM3 treatment and no significant changes with control (Fig. 7J–N, S, T). Although we found no significant difference of total EpETE concentrations from baseline, 14(15)-EpETE was decreased at 1 h followed by an increase at 4 h with P-OM3, returning to baseline by 8 h (Fig. 7Q). Although 17(18)-EpETE also appears to peak at 2–4 h, changes from baseline were not significant (Fig. 7R).

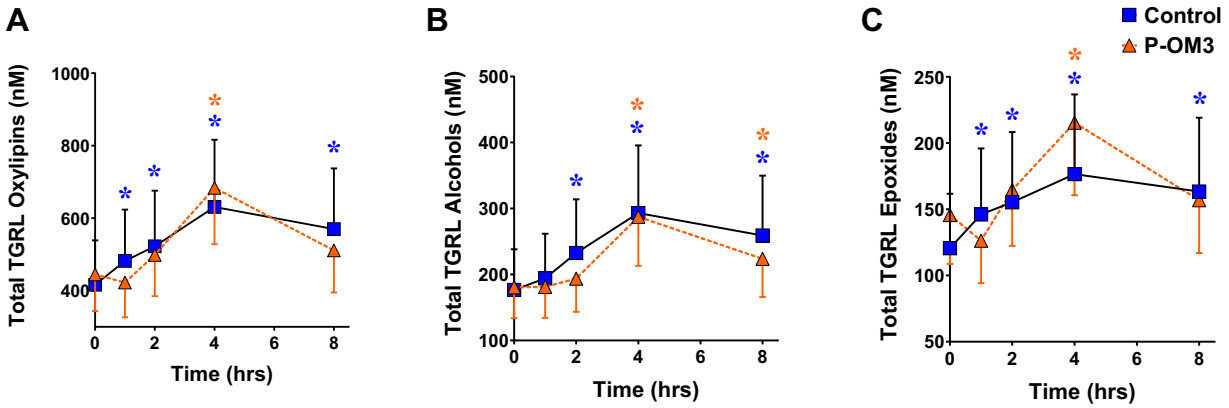

Fig. 5. Response of TGRL oxylipins to endotoxin challenge. Oxylipin concentrations from 0 to 8 h after endotoxin challenge of (A) total oxylipins, (B) total alcohols, and (C) total epoxides. Blue squares represent control treatment; orange triangles represent P-OM3 treatment. Differences were assessed by repeated measures ANOVA with mixed models. Post hoc analysis was done using the False Discovery Rate Correction term, q, with the acceptable false discovery rate of 10% (39). Longitudinal data is graphed as least-squares means ±95% confidence interval. * $P < 0.05$ adjusted for false discovery rate, q = 0.1; Different from baseline time point. P-OM3, prescription ω-3 acid ethyl ester; TGRL, triglyceride-rich lipoprotein.

DISCUSSION

This study confirmed that plasma TGRL oxylipin composition changes with time following endotoxin challenge, consistent with TGRL oxylipins participating as a part of the acute inflammatory response. Alcohols as a class were more abundant than epoxides following endotoxin challenge, but both classes had a similar change from baseline to peak concentrations. The timing of response was different between oxylipins and parent fatty acids. Specifically, both AA and LA alcohols increased in response to endotoxin, but the peak AA-derived HETE response at 2 h precedes that of the LA-derived HODEs at 4 h. The AA-derived epoxides, EpETrEs, followed both these responses with a peak at 8 h, but this interaction did not reach significance. By contrast, LA remained unchanged in response to endotoxin, and AA was moderately lower at 8 h. This suggests that the TGRL incorporation rate of specific oxylipins into lipoproteins during the inflammatory response is upregulated, even while the parent fatty acid content remains stable or decreases. This is consistent with a controlled response that incorporates specific oxylipins into VLDL for delivery to tissues.

Instead of blunting the pro-inflammatory VLDL oxylipin response, P-OM3 altered the types of fatty acids and oxylipins available within the acute response. P-OM3 treatment was associated with increased EPA and DHA enrichment in the TGRL fraction at baseline and across all time points. Surprisingly, although the ω-6 fatty acids AA and LA did not increase after endotoxin challenge, there was an increase in DHA enrichment from baseline at 1–2 h in response to the endotoxin challenge, followed by increased EPA, EPA alcohols, and DHA epoxides at 4 h in the P-OM3 group. Therefore, in addition to regulating oxylipin content in VLDL, it appears that the liver may also preferentially enrich VLDL with ω-3 fatty acids during acute inflammation when they are available, resulting in a lipid profile that facilitates effective inflammatory resolution.

Our finding that peak plasma TG and TGRL oxylipin content occurred at 4 h is similar to other human studies that found peak VLDL lipid content between 3 and 4 h. This is most likely due to increased NEFA released from adipose tissue and available for TG synthesis (23, 24). Animal studies have suggested that VLDL ApoB increases to a greater extent than VLDL lipids in response to endotoxin (26, 27). In contrast, we observed a modest increase in plasma TG peaking at 4 h after endotoxin challenge with no significant pattern in the ApoB 100 response. This suggests that the hyperlipidemic response to endotoxin in humans is primarily a lipid-synthesis response and not an ApoB response. However, it is also notable that the TG and ApoB responses of each individual varied widely, as shown in the supplemental material. Unlike animal studies, subjects in this study were not fasted for the duration of the test, which may explain some of this variation.

P-OM3 treatment did not blunt the plasma TG response to endotoxin in the first 8 h, suggesting that the primary effect of P-OM3 is to stabilize the resolution process, rather than decrease the acute inflammatory response. It is possible that this lack of effect could be explained by the beneficial and potentially anti-inflammatory effects of the olive oil control capsules and their high monounsaturated fatty acid content (40–42), resulting in anti-inflammatory effects of both treatments. However, the amount of olive oil in the control capsules was quite small (4 g olive oil/day; 3.2 g MUFA/day) compared with the habitual average intake of the U.S. population (29 g MUFA/day) (43), so it is not likely to have a potent effect. Additionally, although the control capsules had high OA content, the TGRL OA content was not significantly different by treatment in this study.

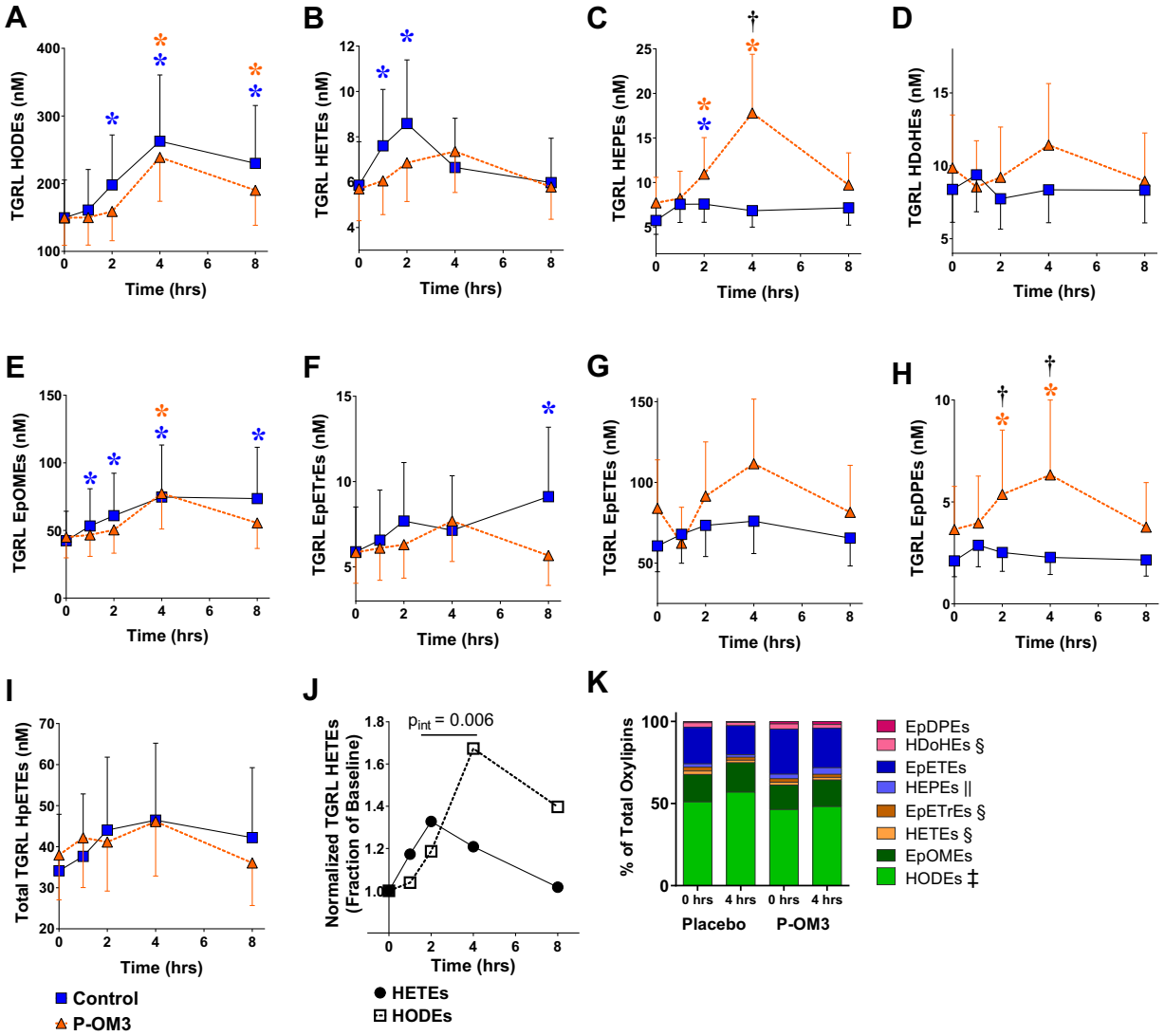

Fig. 6. Effect of P-OM3 treatment on oxylipin response to endotoxin challenge. Least-squares means are shown from 0 to 8 h after endotoxin challenge for (A) linoleic acid (LA)–derived alcohols (HODEs), (B) arachidonic acid (AA)–derived alcohols (HETEs), (C) eicosapentaenoic acid (EPA)–derived alcohols (HEPEs), (D) docosahexaenoic acid (DHA)–derived alcohols (HDoHEs), (E) LA-derived epoxides (EpOMEs), (F) AA-derived epoxides (EpETrEs), (G) EPA-derived epoxides (EpETEs), (H) DHA-derived epoxides (EpDPEs), and (I) AA-derived hydroperoxides (HpETEs). J: The time-dependent response of HODEs differed from that of HETEs with an interaction *P*-value of 0.007. K: Fractional increase in HODEs and fractional decrease in HDoHEs, EpETrEs, and HETEs from 0 to 4 h occurred regardless of treatment. Fractional increase in HEPEs occurred only with P-OM3 treatment. Blue squares represent control treatment; orange triangles represent P-OM3 treatment. Differences were assessed by repeated measures ANOVA with mixed models. Post hoc analysis was done using the False Discovery Rate Correction term, q, with the acceptable false discovery rate of 10% (39). Longitudinal data is graphed as least-squares means ±95% confidence interval. * $P < 0.05$ adjusted for false discovery rate, q = 0.1; Different from baseline time point. † $P < 0.05$ adjusted for false discovery rate, q = 0.1; P-OM3 different from control. ‡ $P < 0.05$ adjusted for false discovery rate, q = 0.1; Increased from 0 to 4 h. § $P < 0.05$ adjusted for false discovery rate, q = 0.1; Decreased from 0 to 4 h. ‖ $P < 0.05$ adjusted for false discovery rate, q = 0.1; Increased from 0 to 4 h only with P-OM3. EpETrE, epoxyeicosatrienoic acid; EpDPE, epoxydocosapentaenoic acid; HDoHE, hydroxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid; HpETE, hydroperoxyeicosatetraenoic acid; P-OM3, prescription ω-3 acid ethyl ester.

P-OM3 treatment also resulted in an increased response of TGRL HEPEs, derived from EPA, and EpDPEs, derived from DHA. The HEPE response was particularly strong, with a peak concentration well over double than at baseline. This supports previous observations in the nonesterified oxylipin fraction that 18-HEPE shows a particularly strong time-dependent increase following endotoxin challenge with P-OM3 treatment compared to control (33). In our data, we found increased concentrations of 5-, 8-, 11-, and 18-HEPE at 4 h after endotoxin challenge. After applying the false discovery rate penalization with an acceptable false positive rate of 10%, 12-HEPE did not show a significant change from baseline, but the concentration was higher with P-OM3 than with control at 4 h. However, using an exploratory *t* test *P*-value (<0.05), 12-HEPE was significantly increased from baseline at 4 h in the P-OM3 group.

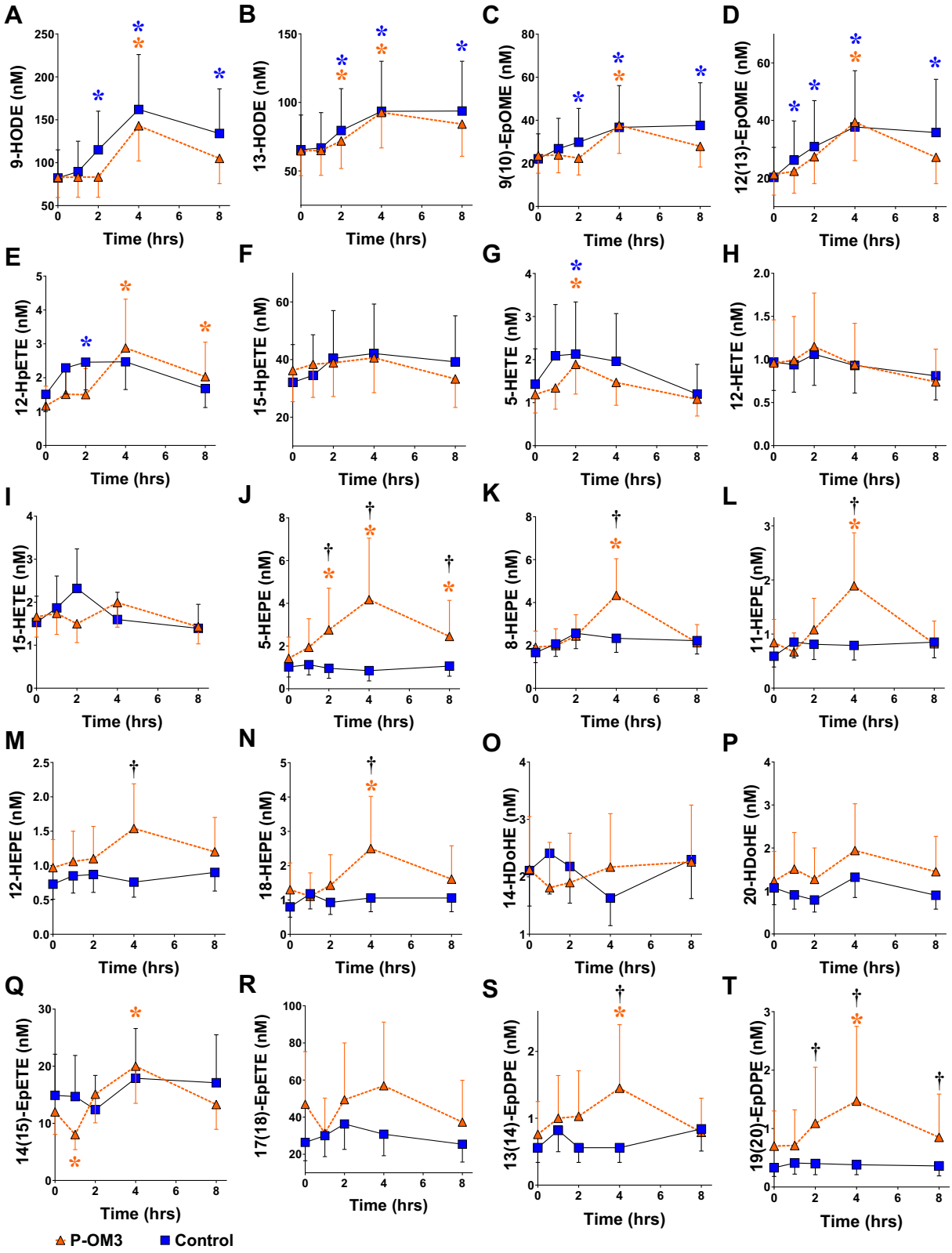

Fig. 7. The time-dependent effect of LPS on individual oxylipins. Measured oxylipins include (A) linoleic acid (LA)–derived 9-HODE, (B) LA-derived 13-HODE, (C) LA-derived 12(13)-EpOME, (D) LA-derived 9(10)-EpOME, (E) arachidonic acid (AA)–derived 12-HpETE, (F) AA-derived 15-HpETE, (G) AA-derived 5-HETE, (H) AA-derived 12-HETE, (I) AA-derived 15-HETE, (J) eicosapentaenoic acid (EPA)–derived 5-HEPE, (K) EPA-derived 8-HEPE, (L) EPA-derived 11-HEPE, (M) EPA-derived 12-HEPE, (N) EPA-derived 18-HEPE, (O) docosahexaenoic acid (DHA)–derived 14-HDoHE, (P) DHA-derived 20-HDoHE, (Q) EPA-derived 14(15)-EpETE, (R) EPA-derived 17(18)-EpETE, (S) DHA-derived 13(14)-EpDPE, and (T) DHA-derived 19(20)-EpDPE. Blue squares represent control treatment; orange triangles represent P-OM3 treatment. Differences were assessed by repeated measures ANOVA with mixed models. Post hoc analysis was done using the False Discovery Rate Correction term, q, with the acceptable false discovery rate

The metabolic syndrome is a condition of chronic low-grade inflammation that is associated with obesity, insulin resistance, and dyslipidemia (44). Chronic endotoxemia with low-grade inflammation from high fat diet has been shown to increase insulin resistance in mice and may contribute to the metabolic syndrome (45). Beneficial changes in the oxylipin response to endotoxemia in TGRL may help to explain the beneficial effects of P-OM3 treatment in hyperlipidemia. We found that P-OM3 increases ω-3 fatty acid incorporation into TGRL in the place of some ω-6 fatty acid content, corresponding with increased levels of both HEPEs and EpDPEs in TGRL. TGRL could therefore be serving to provide anti-inflammatory signals to the peripheral tissue in two ways. First, they deliver preformed ω-3 oxylipin signals directly. Second, they deliver additional ω-3 parent fatty acids (EPA and DHA) so that peripheral tissues can synthesize their own anti-inflammatory oxylipin signals intracellularly as needed.

Strengths and limitations

The use of a low dose of endotoxin (0.6 ng/kg body weight) to elicit an inflammatory response instead of the traditional moderate dose (2 or 3 ng/kg body weight) (23, 24) makes this study generalizable to milder inflammatory conditions than severe infection and overt sepsis (34). We measured lipid-specific responses to observe inflammation effects that may contribute to dyslipidemia. Multiple time-dependent samples provide a detailed understanding of the TGRL fatty acid and oxylipin response to endotoxin, which has not been previously characterized. Using the crossover design allows subjects to be their own treatment controls, which improves power to observe the effects of P-OM3 treatment.

The study also had several limitations. With the exception of the screening and baseline (0 h) measurements, subjects were not fasted during the study, which limits our ability to separate VLDL response from chylomicrons and the effect of inflammation from postprandial meal effects. Also, the sample size limits power to detect small changes. Because of these specific constraints, potential for generalization outside of the current study context is limited, but future studies could be designed to measure the lipid response more precisely. The control used in this trial was olive oil which includes bioactive components that could have health effects that were not neutral. Although the amount of olive oil used in this trial was quite small, we cannot rule out some effect of olive oil in the control treatment. We used sequential density ultracentrifugation in subjects with very low baseline plasma TGs; hence, several oxylipins were close to the limit of detection of our LC-MS/MS methodology, which limited the oxylipins we were able to detect and analyze.

CONCLUSIONS

In this study, multiple oxylipins derived from LA, AA, EPA, and DHA were enriched in the TGRL fraction in the first 8 h following low-dose endotoxin challenge in a time-dependent pattern, even though the ω-6 parent fatty acids LA and AA did not increase. Fractional concentrations of both EPA and DHA increased in the first 8 h after endotoxin challenge, while fractional AA was lower at 8 h. Oxylipin concentrations peaked at 4 h, with the exception of HETEs, which peaked at 2 h, and EpETrEs, which peaked at 8 h. These results support a novel finding that lipoprotein-carried oxylipins are differentially regulated to contribute to both the acute initiation of the inflammatory response and its resolution following an endotoxin challenge. P-OM3 treatment increased the time-dependent response of EPA-derived HEPEs and DHA-derived EpDPEs to endotoxin challenge. However, P-OM3 did not blunt the overall oxylipin response to endotoxin challenge, even in ω-6-derived oxylipins, indicating that P-OM3 treatment primarily acts by increasing ω-3-derived products in TGRL and not by decreasing the response of ω-6-derived products. Therefore, this data suggests that suboptimal concentrations of EPA and DHA may result in lipoprotein oxylipin profiles that are not ideal for resolving the inflammatory response following an acute challenge.

Data availability

Dataset is available upon request. Data requests should be addressed to Gregory Shearer, Department of Nutritional Sciences, the Pennsylvania State University (gcs13@psu.edu).

Supplemental data

This article contains supplemental data.

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Author contributions

R. E. W., C. K. R., A. C. S.-R., M. R. F., B. A. H., and C. E. A. investigation; R. E. W. and G. C. S. formal analysis; R. E. W. writing–original draft; R. E. W. visualization; C. K. R., A. C. S.-R., M. R. F., P. M. K.-E., G. L. J., and G. C. S. writing–review and

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editing; A. C.-S. R., G. L. J., and G. C. S. conceptualization; P. M. K. E., G. L. J., and G. C. S. resources; P. M. K. E. and G. C. S. supervision; G. L. J. funding acquisition.

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Conflict of interest
The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations
AA, arachidonic acid; CRC, clinical research center; EpDPE, epoxydocosapentaenoic acid; EpETE, epoxyeicosatetraenoic acid; EpETrE, epoxyeicosatrienoic acid; EpOME, epoxyoctadecamonoenoic acid; HEPE, hydroxyeicosapentaenoic acid; HpETE, hydroperoxyeicosatetraenoic acid; LA, linoleic acid; LPS, lipopolysaccharide; NEFA, non-esterified fatty acid; P-OM3, prescription ω-3 acid ethyl ester; TG, triglyceride; TGRL, triglyceride-rich lipoprotein.

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