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David C. Nieman, Mary Pat Meaney, Casey S. John, Kevin J. Knagge, Huiyuan Chen

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9- and 13-hydroxy-octadecadienoic acids (9+13 HODE) are inversely related to granulocyte colony stimulating factor and IL-6 in runners after 2 h running

Running Head: HODEs and exercise-induced inflammation

Authors: David C. Nieman[1], Mary Pat Meaney[1], Casey S. John[1], Kevin J. Knagge[2], Huiyuan Chen[2].

[1]Appalachian State University, North Carolina Research Campus, Kannapolis, NC

[2] Analytical Sciences, David H. Murdock Research Institute, Kannapolis, NC

COMMUNICATION:

David C. Nieman, DrPH, North Carolina Research Campus

600 Laureate Way, Kannapolis, NC 28081; Email: niemandc@appstate.edu

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Abstract

This study utilized a pro-inflammatory exercise mode to explore potential linkages between increases in 9- and 13-hydroxy-octadecadienoic acid (9+13 HODE) and biomarkers for inflammation, oxidative stress, and muscle damage. Male (N=10) and female (N=10) runners ran at ~70% VO_{2max} for 1.5 h followed by 30 min of downhill running (-10%). Blood samples were taken pre-run and immediately-, 1-h-, and 24-h post-run, and analyzed for 9+13 HODE, F_2-isoprostanes, six cytokines, C-reactive protein (CRP), creatine kinase (CK), and myoglobin (MYO). Gender groups performed at comparable relative heart rate and oxygen consumption levels during the 2-h run. All outcome measures increased post-run (time effects, $P \leq 0.001$), with levels near pre-run levels by 24 h except for CRP, CK, MYO, and delayed onset of muscle soreness (DOMS). Plasma 9+13 HODE increased 314±38.4% post-run ($P<0.001$), 77.3±15.8% 1-h post-run ($P<0.001$), and 40.6±16.4% 24-h post-exercise ($P=0.024$), and F_2-isoprostanes increased 50.8±8.9% post-run ($P<0.001$) and 19.0±5.3% 1-h post-run ($P=0.006$). Post-run increases were comparable between genders for all outcomes except for 9+13 HODE (interaction effect, $P=0.024$, post-run tending higher in females), IL-10 ($P=0.006$, females lower), and DOMS ($P=0.029$, females lower). The pre-to-post-run increase in 9+13 HODEs was not related to other outcomes except for plasma granulocyte colony stimulating factor (GCSF) ($r = -0.710$, $P<0.001$) and IL-6 ($r = -0.457$, $P=0.043$). Within the context of this study, exercise-induced increases in 9+13 HODEs tended higher in females, and were not related to increases in F_2-isoprostanes, muscle damage, or soreness. The negative relationships to GCSF and IL-6 suggest a linkage between 9+13 HODES and exercise-induced neutrophil chemotaxis, degranulation, and inflammation.

KEYWORDS: running, cytokines, inflammation, oxidative stress, muscle damage, soreness

INTRODUCTION

Linoleic acid, an essential polyunsaturated fatty acid (PUFA), is the direct precursor to oxidized linoleic acid metabolites (OXLAMs) including 9- and 13-hydroxy-octadecadienoic acid (9+13 HODE) (Halliwell et al., 1996; Kokatnur et al., 1979; Ramsden et al., 2012; Spiteller, 1998). 9+13 HODE elicit pleiotropic effects that are either beneficial or detrimental depending on the context (Wittwer and Hersberger, 2007), and are generated by a variety of cell types through several pathways involving 15-lipoxygenase-1 (15-LOX), cyclooxygenases 1 and 2 (COX-1, COX-2), cytochrome P450 microsomal enzymes, and free radical and singlet oxygen oxidations (ROS) (Lehmann et al., 1992; Pasman et al., 2013; Spiteller, 1998). 9+13 HODE are stable and abundant oxidation products that can be measured in human plasma, and have been linked to a wide variety of pathological conditions (Jira et al., 1998; O'Flaherty et al., 2013; Ramsden et al., 2012; Vangaveti et al., 2010, 2015). Elevated levels of plasma 9+13 HODE can be reduced through the adoption of healthy diets or weight loss, and have emerged as important indicators of oxidative stress (Collino et al., 2013; Dandona et al., 2001; Gaskins et al., 2010; Pasman et al., 2013).

In three previous papers utilizing a metabolomics approach, our research group reported that plasma 9+13 HODE increased significantly following prolonged and intensive exercise (Nieman et al., 2013, 2014c, 2015). In one of these studies, increases in plasma 9+13 HODE (median scaled intensity or MSI units) and F_2-isoprostanes (pg/ml) following 75-km cycling were related ($r=0.75$, $P<0.001$), but without significant correlations to increases in six cytokines (Nieman et al., 2014c). This was an unexpected finding due to the ascribed roles of 9+13 HODE in the regulation of inflammation linked to atherogenesis, cancer, the metabolic syndrome, and other pathological processes (Bojic et al., 2016; O'Flaherty et al., 2013; Vangaveti et al., 2010,

2015). 9+13 HODE play a role in signaling and the regulation of inflammatory processes involving cell adhesion molecules, neutrophil chemotaxis and degranulation, macrophage superoxide production, peroxisome proliferator activated receptor gamma (PPAR-γ) activation, and inhibition of protein kinase C (Futosi et al., 2013; Itoh et al., 2008; Smith et al., 2014; Vangaveti et al., 2010; Wittwer and Hersberger, 2007).

With intensive, prolonged exercise, granulocyte blood concentrations increase 3 to 6-fold, and then move into involved muscle tissues where phagocytosis, production of ROS and degranulation occur (Nieman et al., 2014a,b; Peake et al., 2015). Granulocyte degranulation is characterized by the release of hydrolytic and proteolytic enzymes, and the expression of receptors and proteins on the plasma membrane that come from granule membranes and intracellular vesicles. Control of degranulation is important to assure that the granulocyte is activated only at the right place and time and to the proper extent to avoid excessive muscle cell damage (Futosi et al., 2013; Vangaveti et al., 2015). Inflammatory cells such as granulocytes and macrophages convert linoleic acid into HODES, and studies indicate that HODES help regulate degranulation, ROS production, and chemotactic activity (Vangaveti et al., 2010, 2015).

Given the expected role of 9+13 HODE in the regulation of inflammation during exercise, this study employed high intensity running for 1.5 h followed by 30 min of downhill running, an exercise protocol known to induce more muscle damage and inflammation than cycling (Nieman et al., 2014a). Blood samples and delayed onset of muscle soreness (DOMS) ratings were taken pre-run and immediately-, 1-h-, and 24-h post-run. Blood samples were analyzed for 9+13 HODE using high performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (LC-MS/MS), and exercise-induced changes

were tested for associations with F_2-isoprostanes (oxidative stress), six cytokines and C-reactive protein (CRP) (inflammation), and muscle damage biomarkers (creatine kinase and myoglobin).

MATERIALS AND METHODS

Participants. Recruitment was conducted through direct messages to runners in the Charlotte, NC, metropolitan area. Participants included 20 male and female runners (ages 22-45 years) who regularly competed in long-distance road races and were capable of running for two hours on treadmills at ~70% VO_{2max}. Participants consented to train normally, maintain weight, and avoid the use of large-dose vitamin and mineral supplements (above 100% Daily Value), herbal supplements, and medications (in particular, non-steroidal anti-inflammatory drugs) for the 6-week period separating the two lab sessions. Participants signed informed consent forms and study procedures were approved by the Institutional Review Board at Appalachian State University (ASU).

Baseline Testing. Prior to the study, participants were tested for VO_{2max} during a graded, treadmill test with the Cosmed Fitmate metabolic system (Cosmed, Rome, Italy) (Nieman et al., 2013, 2014a). Body composition was measured with the Bod Pod body composition analyzer (Life Measurement, Concord, CA). Demographic and training histories were acquired with questionnaires.

Running Trials. During the 3-day period prior to each running session (separated by six weeks), participants tapered exercise training and followed a moderate-carbohydrate dietary regimen (~55% kcal as carbohydrates) by choosing foods from a list provided by the investigative team (Nieman et al., 2014b, 2015). Participants reported to the lab at 6:45 am in an overnight fasted state, and provided blood samples and a rating of muscle soreness (DOMS) (Smith et al.,1993). At 7:00 am, participants ran on laboratory treadmills with the speed set at

~70% of VO_{2max} for 1.5 hours followed by 30 minutes of downhill running on a -10% grade at close to the same intensity. Participants ingested 2 to 3 ml/kg water every 15 minutes, without ingestion of any other beverages or food. Metabolic measures from the Cosmed Fitmate metabolic system and the rating of perceived exertion (RPE) were taken every 30 minutes during the running bout to verify that the appropriate intensity was maintained. Additional blood and DOMS ratings were taken immediately following the 2-h run, and then 1-h post-run, and 24-h post-run. The blood samples were collected into sterile vacuum blood collection tubes and centrifuged as recommended by the manufacturer. All of these procedures were repeated six weeks later, with runners following the exact same schedule (including time of day and day of the week). The total distances run during the two treadmill sessions were highly correlated ($r=0.984$, $P<0.001$), and data were combined for this analysis to strengthen data quality, reduce random measurement error, and provide better estimates of the "true" value for individual participants (Hecksteden et al., 2015).

9+13-HODE. 9-HODE, 13-HODE, and 9-HODE-d4 standards were purchased from Cayman Chemical (Ann Arbor, MI), methanol, acetonitrile and 2-propanol from Avantor Performance Materials Inc. (Center Valley, PA), ethanol and butylhydroxytoluene (BHT) from Sigma-Aldrich (St. Louis, MO), and acetic acid and hexane from Fisher Scientific (Pittsburgh, PA). All solvents and chemicals used were of HPLC grade or higher. Water was produced by Milli-Q water purification system (Millipore, Billerica, MA). The 9-HODE and 13-HODE stock solutions were 200 ng/mL, and were further diluted with ethanol (containing 50 ng/mL 9-HODE-d4) at eight different calibration levels and stored at −20°C. 9-HODE and 13-HODE from plasma samples were extracted using liquid-liquid extraction. The deuterated compound 9-HODE-d4 was used as an internal standard and added prior to extraction.

Plasma was collected from heparinized blood, flash-frozen in liquid nitrogen, and stored at -80°C before analysis. The entire sample preparation process was then performed on each sample. 10 µL of 500 ng/mL 9-HODE-d4 and 10 µL of 50 mM BHT were added to 200 µL of plasma sample. Then 1.0 mL (10% v/v acetic acid in water)/2-propanol/hexane (2/20/30, v/v/v) and 2.0 mL hexane were used for extraction. After centrifugation, the upper layer was removed, dried and reconstituted with 100 µL of 85% methanol in water (v/v, containing 0.1% acetic acid) for LC-MS analysis (Levison et al., 2013; Rago and Fu, 2013). Chromatographic separation of 9-HODE and 13-HODE was achieved using an UPLC system (Acquity UPLC, Waters, Milford, MA) equipped with Waters BEH C18 1.7 µm analytical column (2.1 × 100 mm). The mobile phases consisted of (A) 0.2% acetic acid in water and (B) 0.2% acetic acid in acetonitrile. The gradient used was: 0-0.5 min (80% B), 0.5-3 min (80-100% B), 3-6 min (100% B), 6-6.1 min (100-80% B), 6.1-7 min (80% B). For detection of 9-HODE and 13-HODE, the UPLC system was coupled with Quattro Premier XE MS (Waters, Milford, MA) and the system was operated in electrospray ionization (ESI) negative mode. The multiple reaction monitoring (MRM) was performed with optimized transitions (295>171 for 9-HODE; 295>195 for 13-HODE; and 299>172 for 9-HODE-d4). All peaks were integrated using TargetLynx Application Manager (Waters, Milford, MA), and the peaks were quantified using calibration curves of peak areas of native compounds divided by the internal standard peak area.

F_2-isoprostanes. Plasma was collected from heparinized blood, flash-frozen in liquid nitrogen, and stored at -80°C before analysis. 8-iso prostaglandin F_2, 8-iso prostaglandin F_2-d4, and prostaglandin F_2 methyl ester were purchased from Cayman Chemical (Ann Arbor, MI). Plasma tubes were labeled and 2 mL water and 20 µL internal standard 8-iso prostaglandin $F_2\alpha$-d4 work solution were added to each tube. 500 µL of plasma sample was added to the tubes and

vortexed, with the pH of the samples adjusted to 3 using 1N HCl. The C-18 Sep-Pak cartridge and Silica Sep-Pak cartridge were purchased from Waters (Milford, MA). One C-18 Sep-Pak cartridge was connected to a 10 mL disposable syringe for each sample and the cartridge was preconditioned with 5 mL methanol and 7 mL water (adjusted to pH 3 with 1N HCl). The sample was applied to the preconditioned Sep-Pak cartridge slowly, and the cartridge was washed twice, first with 10 mL pH 3 water and then with 10 mL heptane. F_2-isoprostanes were eluted from the cartridge with 10 mL ethyl acetate/heptane (50:50, v/v) into a vial, and 5 g of anhydrous sodium sulfate was added. The used C-18 Sep-Pak cartridges were then removed from the syringes and replaced with one silica Sep-Pak cartridge for each sample and preconditioned with 5 mL of ethyl acetate. The eluent from C-18 Sep-Pak cartridge was applied to the silica Sep-Pak cartridge. The cartridge was washed with 5 mL of ethyl acetate. F_2-isoprostanes were eluted from the silica Sep-Pak cartridge with 5 mL ethyl acetate/methanol (1:1, v/v) into a vial. The eluent was evaporated under nitrogen at 37°C. 40 µL of 10% (v/v) PFBB in acetonitrile and 20 µL 10% (v/v) DIPE in acetonitrile were added to each vial, vortexed briefly and incubated for 20 minutes at 37°C. The samples were dried thoroughly under nitrogen flow for about 5 minutes. Each sample was reconstituted in 50 µL methanol/chloroform 3/2 (v/v) and vortexed.

The TLC tank was prepared by adding 93 mL chloroform and 7 mL ethanol. Each sample was applied to a silica TLC plate, with 5 µL of the prostaglandin $F_2\alpha$ methyl ester applied to one lane for use as a standard using a separate TLC plate. TLC plates were placed into the TLC tank and removed from the tank when the solvent front reached 13 cm on the TLC plate. The TLC standard was visualized by spraying the standard plate with the phosphomolybdic acid solution and then heating on a hot plate for 2 minutes. The silica from the sample TLC plates in the region of the TLC standard was scraped from 1 cm above the middle of the visualized

standard to 1 cm below the standard into separate centrifuge tube and 1 mL ethyl acetate was added to each tube, vortexed, then centrifuged at 10,000g for 5 minutes. The ethyl acetate was carefully removed and transferred into a GC glass vial and dried under nitrogen for 20 minutes or until evaporated. 20 µL BSTFA and 8 µL dry DMF were added, and the sample was vortexed and incubated at 37ºC for 5 minutes. The sample was reconstituted in 30 µL dry undecane and vortexed briefly for gas chromatography mass spectrometry (GC-MS) analysis (Milne et al., 2007; Roberts and Milne, 2009).

Samples were analyzed by a negative ion chemical ionization GC-MS using an Agilent 7890A gas chromatography interfaced to an Agilent 5975C inert MSD mass spectrometer (Agilent Technologies, Santa Clara, CA). The GC column used was an Agilent DB1701 column: 15 m (length) × 250 µm I.D., 0.25-µm film thickness. The column temperature was programmed from 190 °C to 300 °C at 20 °C per min. Helium was used as the carrier gas and methane was used as the reagent gas. The ion source temperature was 200 °C and MS quad temperature was 150 °C. The ion monitored for F_2-isoprostanes was m/z 569 and for deuterated internal standard was m/z 573. The raw data files generated by GC-MS were processed using the MSD ChemStation software package (Agilent Technologies., Santa Clara, CA). For quantification purposes, the area of the peak containing derivatized 8-iso prostaglandin F_2 (m/z 569) was compared with the area of 8-iso prostaglandin F_2-d4 peak (m/z 573). Levels of F_2-isoprostanes in plasma are reported in picograms per milliliter. Calculation: (area of m/z 569) / (area of m/z 573) = concentration of 8-iso prostaglandin F_2 / 8-iso prostaglandin F_2-d4.

Cytokines. Plasma was collected from EDTA-treated blood, flash-frozen in liquid nitrogen, and stored at -80°C before analysis. Total plasma concentrations of six inflammatory cytokines [monocyte chemoattractant protein-1 (MCP-1), interleukin 1 receptor antagonist (IL-

1ra), granulocyte colony-stimulating factor (GCSF), IL-6, IL-8, and IL-10] were determined using an electrochemiluminescence based solid-phase sandwich immunoassay (Meso Scale Discovery, Gaithersburg,MD, USA). All samples and provided standards were analyzed in duplicate, and the intra-assay CV ranged from 1.7% to 7.5% and the inter-assay CV 2.4 to 9.6% for all cytokines measured. Pre-and post-exercise samples for the cytokines were analyzed on the same assay plate to decrease inter-kit assay variability.

C-reactive protein (CRP), creatine kinase, myoglobin, delayed onset of muscle soreness. CRP and creatine kinase (CK) were measured in fresh serum samples using an AU5800 Clinical Chemistry System (Beckman Coulter Electronics, Brea, CA). Myoglobin (MYO) was measured in fresh serum samples using an electrochemiluminescence immunoassay. Delayed onset of muscle soreness (DOMS) was assessed using the 1–10 scale produced by Smith et al. (1993).

Statistical Analysis. Data analysis was performed using IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp., Armonk, NY). Data from the two running trials were averaged and expressed as mean ± SE. Demographic, metabolic, and performance measures were compared between male (N=10) and female (N=10) runners using independent t-tests, with the alpha level set at $P \leq 0.05$. DOMS and all blood measures were compared between genders using a general linear model (GLM) repeated measures approach [2 (male, female) x 4 (time points)]. Change from pre-run values were contrasted between gender groups for immediate post-run, 1-h post-run, and 24-h post-run time points using independent t-tests at the $P \leq 0.017$ Bonferroni threshold adjustment for multiple comparisons. Post-hoc contrasts from pre-run values were also performed for all subjects combined (N=20) using paired t-tests at the $P \leq 0.017$ Bonferroni threshold adjustment for multiple comparisons. Correlations between 9+13-HODE

and other variables of interest were made using Pearson's product-moment correlation coefficients.

Results

Subject characteristics for the 10 male and 10 female participants are summarized in Table 1. Gender groups had similar age and training volumes, and male compared to female runners had lower body fat percentage and higher VO_{2max} fitness levels.

Table 2 summarizes average metabolic and performance data from the two treadmill run sessions for the first 90 minutes of level grade treadmill running and the final 30 minutes of downhill running on a -10% grade. Male compared to female runners ran further during the entire 2-hour treadmill run, but gender groups performed at comparable relative heart rate and oxygen consumption levels.

Exercise time and interaction (time x gender) effects for outcome measures in the male and female runners are summarized in Table 3, Figure 1 (plasma 9+13 HODE), and Figure 2 (plasma F_2-isoprostanes). The 2-hour run was associated with significant increases in all outcome measures except the ratio of IL-6/IL-10 (all time effects, $P \leq 0.001$), with levels back to near pre-run levels by 24 hours post-exercise except for CRP, muscle soreness, and muscle damage markers (DOMS, creatine kinase, myoglobin). Plasma 9+13 HODE increased 314±38.4% post-run ($P<0.001$), 77.3±15.8% 1-h post-run ($P<0.001$), and 40.6±16.4% 24-h post-exercise ($P=0.024$) (all subjects combined compared to pre-run levels) (Figure 1). Plasma F_2-isoprostanes increased 50.8±8.9% post-run ($P<0.001$) and 19.0±5.3% 1-h post-run ($P=0.006$) (all subjects combined compared to pre-run levels) (Figure 2).

The pattern of increase over time was comparable between gender groups for all outcome measures except for 9-HODE and 13-HODE (both HODEs separately and combined), IL-10, and

DOMS. Pre-to-post-run increases in levels of plasma 9-HODE, 13-HODE, and 9+13-HODE tended to be higher in female compared to male runners, but these were not significant (P=0.069, 0.056, 0.049, respectively) at the P≤0.017 Bonferroni threshold adjustment for multiple comparisons.

The pre-to-post-run increase in 9+13 HODEs for all subjects combined was tested for associations with increases in all other outcome measures listed in Table 3 and Figure 1 and 2. Only two correlations were significant, and these are depicted in Figures 3 and 4. Figure 3 shows the scatterplot relationship between the pre-to-post-run change in plasma 9+13 HODE and plasma granulocyte colony stimulating factor (GCSF) for male and female runners combined (r = -0.710, P<0.001), with Figure 4 showing the relationship for plasma IL-6 (r = -0.457, P=0.043). The post-run increase in DOMS was positively related to the total treadmill running distance (r=0.708, P<0.001), the 2-h run final rating of perceived exertion (RPE) (r=0.866, P<0.001), and the increase in serum myoglobin (r=0.494, P=0.027).

Discussion

This study showed that an intensive 1.5-h run followed by 30 minutes of downhill running induced a 3.1-fold increase in LC-MS/MS quantified plasma 9+13 HODE, with levels still elevated above pre-run levels one day later (41%). In three previous exercise studies utilizing global metabolomics and median-scaled intensity units, post-exercise plasma 9+13 HODE increases ranged from 3.1- to 5.5-fold immediately after 75-km cycling and 7.5 h running over a 3-day period (Nieman et al., 2013, 2014c, 2015). Utilizing a targeted, lipidomics approach, Markworth et al. (2013) reported much smaller increases (~35%) in 9+13 HODE during the first three hours of recovery from one bout of leg resistance exercise (3 sets, 8-10 repetitions, leg squats, leg press, leg extensions) in 16 untrained males.

Immediate post-run increases in plasma 9+13 HODE trended higher in female (4.0-fold) compared to male (2.3-fold) runners (P=0.032), with no gender differences at other recovery times points. No clear consensus has emerged regarding potential gender differences in exercise-induced oxidative stress. In a study of 54 male and female runners competing in the 160-km Western States Endurance Run, no gender differences were measured for post-race increases in plasma and urine F_2-isoprostanes (McAnulty et al., 2007). In the current study, the pattern of increase over time for plasma F_2-isoprostanes was also comparable between gender groups (Figure 2).

The post-run increase in the oxidized linoleic acid metabolites plasma 9+13 HODE was much greater and prolonged than was measured for plasma F_2-isoprostanes. The level of F_2-isoprostanes in plasma or urine is widely regarded as an excellent indicator of exercise-induced oxidative stress and is formed via free radical-initiated peroxidation of arachidonic acid (Milne et al., 2007; Roberts and Milne, 2009). We recently reported that post-75-km cycling plasma levels of 9+13 HODE were positively correlated with postexercise F_2-isoprostanes ($r = 0.75$, $P < 0.001$) and other fatty acids in the linoleate conversion pathway (Nieman et al., 2014c). No significant correlation between plasma 9+13 HODE and F_2-isoprostanes was measured after 2 h running in the current study, which could be explained by several factors including differences in exercise mode, and issues related to the use of global, untargeted metabolomics versus 9+13 HODE quantification by LC-MS/MS. Another issue to consider is the difficulty of measuring the small quantity of F_2-isoprostanes in plasma with GC-MS compared to the relative ease of measuring much higher concentrations of plasma 9+13 HODE using LC-MS/MS (Levison et al., 2013; Nikolaidis et al., 2011). Lipoxygenase products such as 9+13 HODE and hydroxyeicosatetraenoic acids (HETEs), and COX-derived oxidized species like prostaglandin

$F_2\alpha$ predominate in plasma, in comparison to free radical-derived oxidized F_2-isoprostanes that predominate in urine (Levison et al., 2013). Future exercise-related studies should consider measuring oxidative stress with 9+13 HODE from plasma, and F_2-isoprostanes from spot urine samples, as recommended by Levison et al. (Levison et al., 2013).

HODES are considered both a stable indicator of oxidative stress (lipid peroxidation) and a strong regulator of inflammation (Markworth et al., 2013; Niki, 2013; O'Flaherty et al., 2013; Rolin et al., 2014; Spiteller, 1998; Tyurin et al., 2012; Vangaveti et al., 2010, 2015; Viswanathan et al., 2003; Wittwer and Hersberger, 2007). This study utilized a pro-inflammatory exercise mode to explore potential linkages between increases in 9+13 HODE, CRP, muscle damage and soreness, and six cytokines that were selected based on their responsiveness to heavy exertion (Nieman et al., 2014a, 2014b, 2014c, 2015; Peake et al., 2015). These data support a negative relationship between post-exercise 9+13 HODE and two cytokines, GCSF and IL-6, and supports a potential, novel regulatory function for 9+13 HODE within this exercise context. GCSF is produced by endothelium, macrophages, and a number of other immune cells, and stimulates the survival, proliferation, differentiation, and function of neutrophil precursors and mature neutrophils, with regulation in part through protein kinases (Cornish et al., 2009; Eyles et al., 2006; Futosi et al., 2013; Hamilton and Achuthan, 2013). 9+13 HODE help control inflammation through regulation of cell adhesion molecules, neutrophil chemotaxis and degranulation, macrophage superoxide production, PPAR-γ activation, and inhibition of protein kinase C (Futosi et al., 2013; Itoh et al., 2008; Rolin et al., 2014; Vangaveti et al., 2010; Wittwer and Hersberger, 2007). Thus 9+13 HODE may regulate and decrease immune cell release of GCSF to assist in the proper control of neutrophil function, perhaps mitigating excessive neutrophil ROS, degranulation, and muscle cell damage.

Exercise-induced changes in 9+13 HODE were inversely related to increases in plasma IL-6, indicating another potential influence on inflammation regulation. Cell culture studies indicate that HODEs inhibit the release of IL-6 from human monocytes (Rolin et al., 2014). HODEs are natural ligands for PPAR-γ, and may attenuate the release of IL-6 from recruited monocytes by activating PPAR-γ in these cells and others, thereby regulating inflammation at sites of injury and damage, as suggested by Rolin et al. (2014). The IL-6/IL-10 ratio was not significantly changed following the 2-h running bout, suggesting that anti- and pro-inflammatory influences were sufficiently balanced.

In summary, a 1.5-h run followed by 30 minutes of downhill running was associated with significant increases in 9+13 HODE, F_2-isoprostanes, muscle damage and soreness, and six cytokines. A novel, inverse relationship was found between 9+13 HODE and two cytokines, GCSF and IL-6, supporting a linkage between these oxidized derivatives of linoleic acid and post-exercise inflammation. Limitations in this study included the measurement of just one oxidative stress indicator (F_2-isoprostanes), and the lack of muscle and fat biopsy samples to explore these relationships at the tissue level. Additional research is needed to explore the relationship between 9+13 HODE, GCSF, and IL-6 within the context of chronic, exercise training. Useful information would also be gleaned from measuring plasma cytokine and blood granulocyte responses during 9+13 HODE infusion in endurance athletes.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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Figure Legends

Figure 1 Change in plasma 9- and 13-hydroxy-octadecadienoic acid (9+13-HODE) across four time points for male (N=10) and female (N=10) runners: pre-run, immediately post-2-h run, and 1-h and 24-h post-run. Time main effect, P<0.001; time x gender interaction effect, P=0.024.

† P≤0.017, contrast for total subjects combined compared to pre-run (time effects)

Figure 2 Change in plasma F_2-isoprostanes across four time points for male (N=10) and female (N=10) runners: pre-run, immediately post-2-h run, and 1-h and 24-h post-run. Time main effect, P<0.001; time x gender interaction effect, P=0.517.

† P≤0.017, contrast for total subjects combined compared to pre-run (time effects)

Figure 3 Scatterplot relationship between the pre-to-post-run change in plasma 9+13 HODE and plasma granulocyte colony stimulating factor (GCSF) for male and female runners combined (r = -0.710, P<0.001).

Figure 4 Scatterplot relationship between the pre-to-post-run change in plasma 9+13 HODE and plasma IL-6 for male and female runners combined (r = -0.457, P=0.043).

Table 1 Subject characteristics (mean±SE).

Variable	**Males (N=10)**	**Females (N=10)**	**P-value**
Age (years)	36.3±1.8	35.4±2.3	0.765
Height (m)	1.78±0.02	1.62±0.02	<0.001
Weight (kg)	74.5±3.7	55.1±1.6	<0.001
Body fat (%)	12.9±2.2	20.0±2.5	0.046
VO_{2max} ($ml \cdot kg^{-1} min^{-1}$)	57.2±2.7	45.2±2.2	0.003
HR_{max} (beats/min)	177±2.6	181±4.1	0.456
Training (km/wk)	59.2±6.8	53.9±6.8	0.582

Table 2 Average metabolic and performance data after 90 minutes of level grade running and the final 30 minutes of downhill running (-10% grade). Measurements were recorded every 30 minutes during the 2-hour run.

Variable	Males (N=10)	Females (N=10)	P-value
90 min level running			
Distance (km)	17.8±0.7	14.4±0.5	<0.001
30 min -10% grade running			
Distance (km)	6.84±0.30	5.90±0.21	0.021
Total distance (km)	24.7±0.9	20.2±0.6	0.001
90 min level running			
VO_2 ($ml \cdot kg^{-1} min^{-1}$)	40.5±1.7	32.7±1.1	0.001
$\%VO_{2max}$	71.2±1.2	73.0±1.9	0.411
30 min -10% grade running			
VO_2 ($ml \cdot kg^{-1} min^{-1}$)	37.0±1.9	29.8±1.1	0.004
$\%VO_{2max}$	64.8±1.7	66.4±1.8	0.524
90 min level running			
HR (beats/min)	155±2.9	157±4.4	0.660
% HR_{max}	87.6±1.0	87.5±3.2	0.985
30 min -10% grade running			
HR (beats/min)	159±4.6	162±4.1	0.616
% HR_{max}	89.6±1.7	89.9±2.6	0.923
90 min level running			
RPE	13.2±0.4	12.1±0.5	0.087
30 min -10% grade running			
RPE	15.8±0.8	14.4±0.8	0.217

VO_2, volume of oxygen consumed; HR, heart rate; RPE, rating of perceived exertion.

Table 3 Exercise time and interaction (time x gender) effects for outcome measures in male (N=10) and female (N=10) athletes before and after running 2 hours.

Variable		Pre-Run	Post-Run	1-h Post-Run	24-h Post-Run	P-values Time; Interaction
IL-6 (pg/ml)	M	3.17±0.41	11.8±2.23†	9.03±1.41†	2.36±0.31	<0.001;
	F	2.88±0.91	7.46±0.89	7.37±1.56	2.99±1.19	0.105
IL-8 (pg/ml)	M	9.99±1.00	22.4±2.9†	18.1±1.5†	8.12±0.50	<0.001;
	F	8.88±0.68	16.1±1.1	16.6±2.1	9.17±0.68	0.064
IL-10 (pg/ml)	M	2.50±0.18	9.33±1.77†	10.5±1.9†*	2.51±0.53	<0.001;
	F	3.31±0.64	6.20±0.91	6.03±0.81	3.51±0.81	0.006
IL-1ra (pg/ml)	M	111±10.8	216±14.8	385±83.8	111±9.8	<0.001;
	F	125±18.4	215±40.5	300±80.4	117±9.3	0.473
MCP-1 (pg/ml)	M	253±12.9	451±42.3†	487±42.3†	271±18.2	<0.001;
	F	242±15.7	383±27.5	367±32.7	227±12.3	0.130
GCSF (pg/ml)	M	11.0±1.1	17.1±2.0†	18.9±2.0†	10.5±1.0	<0.001;
	F	10.0±1.3	13.9±1.8	17.6±2.4	10.1±1.0	0.433
IL-6/IL-10	M	1.25±0.10	1.53±0.23	1.07±0.18	1.03±0.08	0.389;
	F	1.13±0.38	1.30±0.41	1.29±0.17	1.14±0.43	0.649
DOMS	M	2.08±0.26	6.42±0.54†	6.10±0.43†	5.91±0.53†	<0.001;
	F	1.98±0.36	4.75±0.56	4.55±0.48	5.53±0.40	0.029
CK (μkat/L)	M	189±27.3	259±33.0†	248±27.4†	416±59.2†	<0.001;
	F	147±23.1	222±31.2	227±27.0	343±49.2	0.686
MYO (ng/ml)	M	39.0±2.4	196±22.5†	274±45.7†	70.7±7.3†	<0.001;
	F	31.6±2.8	161±31.8	212±44.6	48.4±7.0	0.475
CRP (mg/L)	M	0.71±0.15	0.67±0.17	0.62±0.16	2.56±0.50†	0.001;
	F	0.70±0.19	0.71±0.22	0.70±0.19	2.02±0.73	0.465
9-HODE (ng/ml)	M	4.66±0.66	13.6±1.6†	8.38±1.08†	6.06±1.17	<0.001;
	F	4.07±0.63	16.8±1.7	6.48±0.95	5.29±0.71	0.038
13-HODE (ng/ml)	M	8.42±093	28.3±3.2†	15.6±2.4†	10.3±2.4	<0.001;
	F	7.75±0.82	38.9±4.9	14.0±2.1	11.7±1.5	0.034

† P≤0.017, contrast for total subjects combined compared to pre-run (time effects)
* P≤0.017, contrast between genders in change from pre-run (interaction effects); IL = interleukin; IL-1ra = interleukin 1 receptor antagonist; MCP-1=monocyte chemoattractant protein-1; GCSF=granulocyte colony-stimulating factor; CK = creatine kinase; CRP = C-reactive protein; HODE = hydroxy-octadecadienoic acids; MYO = myoglobin.

Figure 1

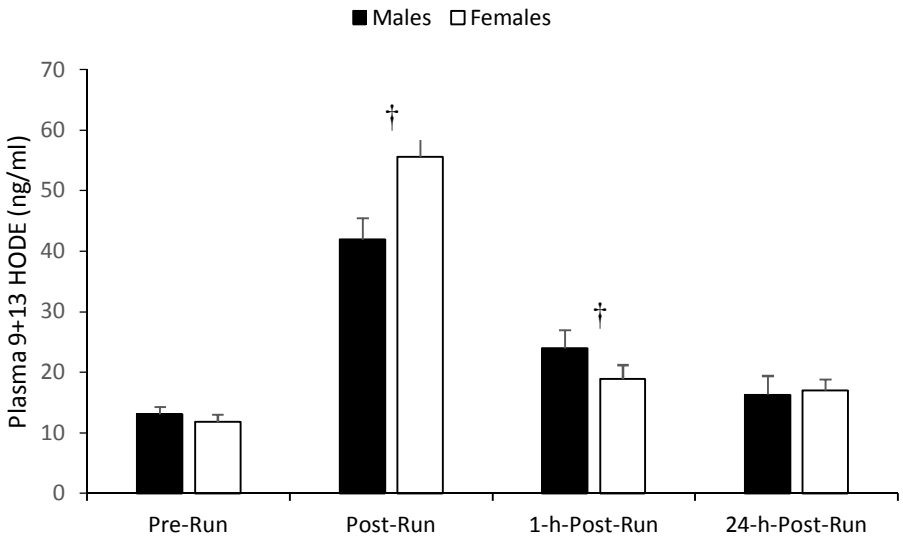


Figure 2

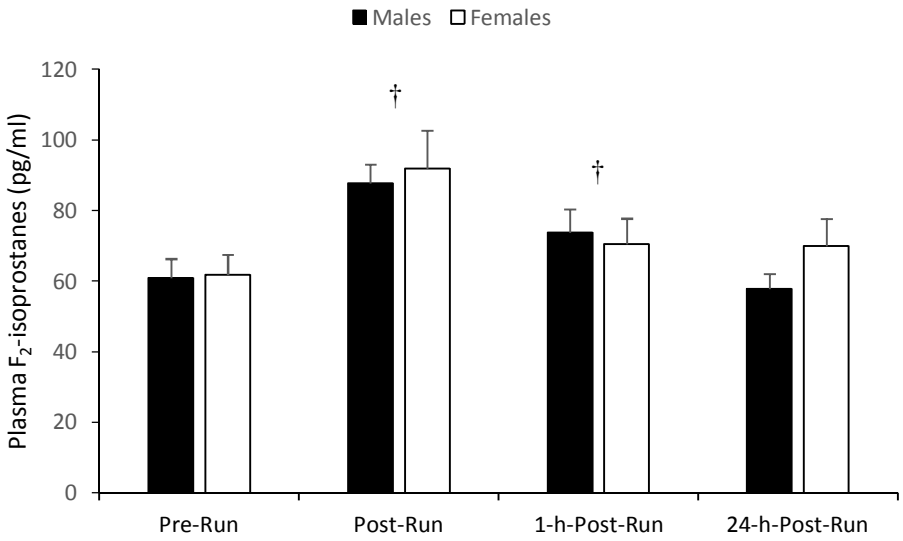


Figure 3

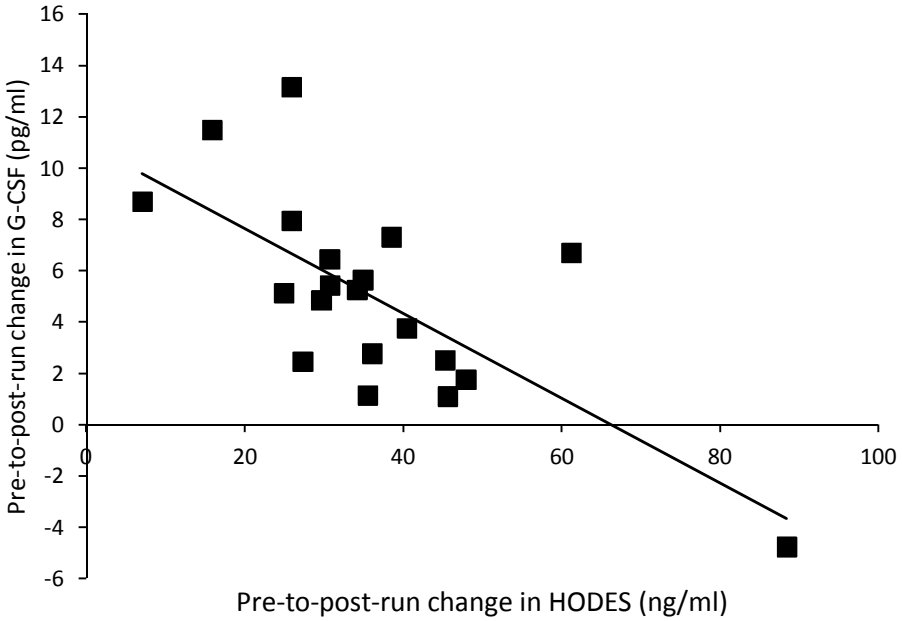
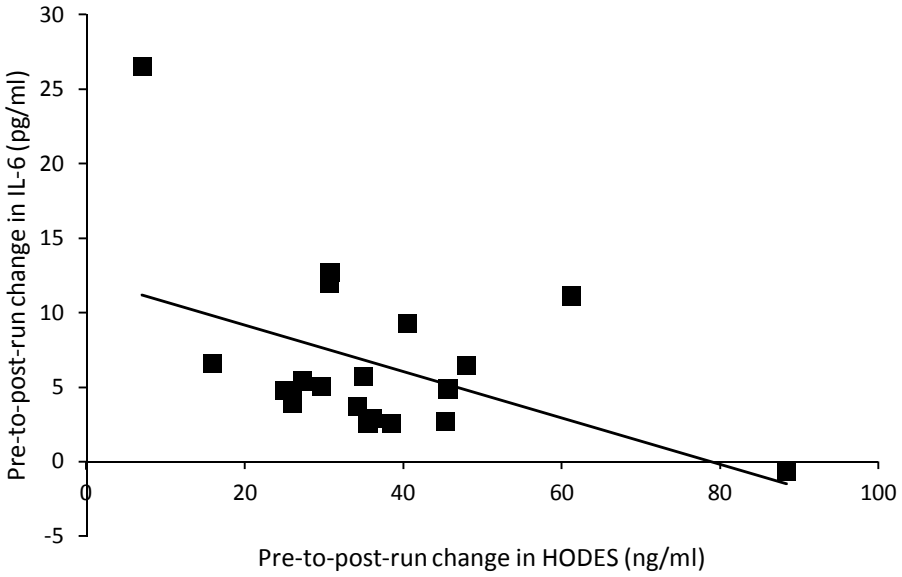


Figure 4



HIGHLIGHTS:

- Linoleic acid, an essential polyunsaturated fatty acid, is the direct precursor to oxidized linoleic acid metabolites including 9- and 13-hydroxy-octadecadienoic acid (9+13 HODE). HODES are considered both a stable indicator of oxidative stress and a strong regulator of inflammation.
- This study utilized a pro-inflammatory exercise mode to explore potential linkages between increases in 9+13 HODE, CRP, muscle damage and soreness, and six cytokines.
- This study showed that an intensive 1.5-h run followed by 30 minutes of downhill running induced a 3.1-fold increase in plasma 9+13 HODE, with levels still elevated above pre-run levels one day later (41%). The post-run increase in 9+13 HODE was much greater and prolonged than was measured for plasma F_2-isoprostanes.
- Data support a negative relationship between post-exercise 9+13 HODE and two cytokines, GCSF and IL-6, and further research is warranted to determine underlying mechanisms for this potential, novel regulatory function for 9+13 HODE within this exercise context.