Metabolomics approach to assessing plasma 13- and 9-hydroxy-octadecadienoic acid and linoleic acid metabolite responses to 75-km cycling

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Abstract

Bioactive oxidized linoleic acid metabolites (OXLAMs) include 13- and 9-hydroxy-octadecadienoic acid (13-HODE + 9-HODE) and have been linked to oxidative stress, inflammation, and numerous pathological and physiological states. The purpose of this study was to measure changes in plasma 13-HODE + 9-HODE following a 75-km cycling bout and identify potential linkages to linoleate metabolism and established biomarkers of oxidative stress (F2-isoprostanes) and inflammation (cytokines) using a metabolomics approach. Trained male cyclists (N ⊠ 19, age 38.0 + 1.6 yr, wattsmax 304 +10.5) engaged in a 75-km cycling time trial on their own bicycles using electromagnetically braked cycling ergometers (2.71 + 0.07 h). Blood samples were collected preexer-cise, immediately post-, 1.5 h post-, and 21 h postexercise, and analyzed for plasma cytokines (IL-6, IL-8, IL-10, tumor necrosis factor-⊠, monocyte chemoattractant protein-1, granulocyte colony-stimulating factor), F2-isoprostanes, and shifts in metabolites using global metabolomics procedures with gas chromatography mass spec-trometry (GC-MS) and liquid chromatography mass spectrometry (LC-MS). 13-HODE +9-HODE increased 3.1-fold and 1.7-fold immediately post- and 1.5 h postexercise (both P <0.001) and returned to preexercise levels by 21-h postexercise. Post-75-km cy-cling plasma levels of 13-HODE ⊠ 9-HODE were not significantly correlated with increases in plasma cytokines but were positively correlated with postexercise F2-isoprostanes (r =0.75, P < 0.001), linoleate (r = 0.54, P = 0.016), arachidate (r = 0.77, P < 0.001), 12,13-dihydroxy-9Z-octadecenoate (12,13-DiHOME) (r = 0.60, P = 0.006), dihomo-linolenate (r =0.57, P = 0.011), and adrenate (r = 0.56, P = 0.013). These findings indicate that prolonged and intensive exercise caused a transient, 3.1-fold increase in the stable linoleic acid oxidation product 13-HODE + 9-HODE and was related to increases in F2-isoprostanes, linoleate, and fatty acids in the linoleate conver-sion pathway. These data support the use of 13-HODE + 9-HODE as an oxidative stress biomarker in acute exercise investigations

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Metabolomics approach to assessing plasma 13- and 9-hydroxy-octadecadienoic acid and linoleic acid metabolite responses to 75-km cycling

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Bioactive oxidized linoleic acid metabolites (OXLAMs) include 13- and 9-hydroxy-octadecadienoic acid (13-HODE + 9-HODE) and have been linked to oxidative stress, inflammation, and numerous pathological and physiological states. The purpose of this study was to measure changes in plasma 13- HODE + 9-HODE following a 75-km cycling bout and identify potential linkages to linoleate metabolism and established biomarkers of oxidative stress (F_2-isoprostanes) and inflammation (cytokines) using a metabolomics approach. Trained male cyclists (N = 19, age 38.0 ± 1.6 yr, $watts_{max}$ 304 ± 10.5) engaged in a 75-km cycling time trial on their own bicycles using electromagnetically braked cycling ergometers (2.71 ± 0.07 h). Blood samples were collected preexercise, immediately post-, 1.5 h post-, and 21 h postexercise, and analyzed for plasma cytokines (IL-6, IL-8, IL-10, tumor necrosis factor-ex, monocyte chemoattractant protein-1, granulocyte colony-stimulating factor), F_2-isoprostanes, and shifts in metabolites using global metabolomics procedures with gas chromatography mass spectrometry (GC-MS) and liquid chromatography mass spectrometry (LC-MS). 13-HODE + 9-HODE increased 3.1-fold and 1.7-fold immediately post- and 1.5 h postexercise (both $P < 0.001$) and returned to preexercise levels by 21-h postexercise. Post-75-km cycling plasma levels of 13-HODE + 9-HODE were not significantly correlated with increases in plasma cytokines but were positively correlated with postexercise F_2-isoprostanes ($r = 0.75$, $P < 0.001$), linoleate ($r = 0.54$, $P = 0.016$), arachidate ($r = 0.77$, $P < 0.001$), 12,13-dihydroxy-9Z-octadecenoate (12,13-DiHOME) ($r = 0.60$, $P = 0.006$), dihomo-linolenate ($r = 0.57$, $P = 0.011$), and adrenate ($r = 0.56$, $P = 0.013$). These findings indicate that prolonged and intensive exercise caused a transient, 3.1-fold increase in the stable linoleic acid oxidation product 13-HODE + 9-HODE and was related to increases in F_2-isoprostanes, linoleate, and fatty acids in the linoleate conversion pathway. These data support the use of 13-HODE + 9-HODE as an oxidative stress biomarker in acute exercise investigations.

exercise; oxidative stress; inflammation; linoleate; metabolites

THE RATE OF WHOLE BODY and muscle oxygen consumption increases during acute, high-intensity, and continuous exercise bouts and is accompanied by an increase in production of reactive oxygen species (ROS) (8, 26, 27). The causes of increased ROS during exercise include electron leakage in the mitochondrial electron transport chain, activation of neutrophils and other phagocytic cells, auto-oxidation of catecholamines, and activation of several enzymes including xanthine oxidase, phospholipase A2, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (8). Direct measurement of free radical and ROS production is difficult, and assessment of oxidative stress during exercise is typically made using indirect methods (22). Reliable oxidative stress biomarkers should be chemically unique and detectable, have relatively long half-lives, and be responsive to increases or decreases in ROS (26). The level of F_2-isoprostanes (F_2-IsoP) in blood 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 (22, 30).

Linoleic acid (18:2n-6) is the most common polyunsaturated fatty acid (PUFA) in human diets and mammalian tissue and is considered essential because humans lack delta-12 and delta-15 desaturase enzymes (9, 15, 29, 33). Ingested linoleic acid is converted to longer and more unsaturated fatty acids through enzymatic desaturation and elongation in the endoplasmic reticulum of cells. Linoleic acid is the direct precursor to oxidized linoleic acid metabolites (OXLAMs) including 13- and 9-hydroxy-octadecadienoic acid (13-HODE + 9-HODE) (9, 15, 29, 33) (see http://www.genome.jp/kegg-bin/show_pathway?map00591 for more detail). 13-HODE + 9-HODE are stable oxidation products and have been linked to pathological conditions including atherosclerosis, diabetes, Alzheimer's disease, non-alcoholic steatohepatitis, psoriasis, chronic inflammation, obesity, and cancer (14, 23, 29, 37). Plasma levels of 13-HODE + 9-HODE are responsive to lifestyle interventions, with decreases reported when subjects adopt healthy diets and lose excess body weight (2, 3, 7, 25). 13-HODE + 9-HODE are generated through the 15-lipoxygenase-1 (15-LOX) pathway in a variety of cell types (17, 25), are ligands of peroxisome proliferator-activated receptors (PPARs) (28), and can act through G protein-coupled receptor 132 (GPR132) to exert pro-inflammatory effects (37).

Little information has been published on the influence of exercise on plasma 13-HODE + 9-HODE, and whether postexercise increases relate to changes in established biomarkers of oxidative stress and inflammation (19, 20). Using a metabolomics approach, we recently reported that plasma 13-HODE + 9-HODE increased 5.5-fold in 15 runners following a 3-day period of intensified training (2.5 h/day running at 70% $\dot{V}O_{2max}$), with levels returning to preexercise levels after 14-h recovery (20). Metabolomics allows the simultaneous measurement of hundreds of metabolites and is especially valuable when focusing on complex interactions within the body during exercise or nutrition interventions. The purpose of this study

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was to measure the effect of prolonged and intensive exercise on plasma 13-HODE + 9-HODE and linoleic acid metabolism and correlations with postexercise increases in plasma F_2-IsoP and inflammatory cytokines.

MATERIALS AND METHODS

Subjects. Subjects included 19 male cyclists (ages 27–49 yr) who regularly competed in road races (racing categories 1 to 5, with 5 assigned to cyclists with the lowest performance ability and experience) and were capable of cycling 75 km at race pace. Subjects trained normally, maintained weight, and avoided the use of large-dose vitamin and mineral supplements (above 100% Daily Value), and all herbal supplements and medications for the 2-wk period before the 75-km cycling time trial. All subjects signed informed consent and all study procedures were approved by the Institutional Review Board at Appalachian State University.

Research design. Two weeks before the 75-km time trial, athletes completed orientation and baseline testing in the North Carolina Research Campus Human Performance Laboratory operated by Appalachian State University in Kannapolis, NC. Demographic and training histories were acquired with questionnaires. Maximal power, oxygen consumption, ventilation, and heart rate were measured during a graded exercise test (25 Watts increase every 2 min, starting at 150 Watts) with the Cosmed Quark CPET metabolic cart (Rome, Italy) and the Lode cycle ergometer (Lode Excaliber Sport, Lode, Groningen, The Netherlands). Body composition was measured with the Bod Pod body composition analyzer (Life Measurement, Concord, CA).

Two weeks after baseline testing, subjects returned to the Human Performance Laboratory at 6:45 AM in an overnight fasted state (no food or beverages other than water for at least 9 hours) and provided a preexercise blood sample. After warming up, subjects cycled on their own bicycles on CompuTrainer Pro model 8001 trainers (RacerMate, Seattle, WA) with heart rate and rating of perceived exertion (RPE) recorded every 30 min and workload continuously monitored using the CompuTrainer MultiRider software system (version 3.0, RacerMate, Seattle, WA). A mountainous 75-km course with moderate difficulty was chosen and programmed into the software system. Oxygen consumption and ventilation were measured using the Cosmed Quark CPET metabolic cart after 16 and 55 km cycling. Subjects were allowed to ingest water ad libitum during the 75-km cycling time trial, but not any other beverage or food containing energy or nutrients. Blood samples were taken via venipuncture immediately and 1.5 h after completing the 75-km time trial. Subjects returned in an overnight fasted state the next morning to provide a 21-h postexercise blood sample. No dietary restrictions were imposed from 1.5-h postexercise to the beginning of the overnight fast.

F_2-isoprostanes. Plasma F_2-IsoP were determined using gas chromatography mass spectrometry (GC-MS) (18). Plasma was collected from heparinized blood, flash-frozen in liquid nitrogen, and stored at -80°C. Plasma samples were thawed, and free F_2-IsoP was extracted with deuterated [$^2H_4$]prostaglandin F_2ex added as an internal standard. Waters Sep-Pak C18 cartridges followed by Waters Sep-Pak Silica cartridges were used for solid phase extraction. F_2-IsoP were converted to pentafluorobenzyl esters, subjected to thin layer chromatography, and converted to trimethylsilyl ether derivatives. Samples were analyzed by a negative ion chemical ionization GC-MS using an Agilent 6890N gas chromatography interfaced to an Agilent 5975B inert MSD mass spectrometer (Agilent Technologies, Santa Clara, CA).

Cytokines. Total plasma concentrations of six inflammatory cytokines [monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-ex (TNF-ex), 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). All samples and provided standards were analyzed in duplicate, and the intra-assay coefficient of variation (CV) ranged from 1.7% to 7.5% and the interassay CV 2.4 to 9.6% for all cytokines measured. Pre- and postexercise samples for the cytokines were analyzed on the same assay plate to decrease interkit assay variability.

Metabolomics. The nontargeted metabolic profiling instrumentation employed for this analysis combined three independent platforms: ultrahigh performance liquid chromatography/tandem mass spectrometry (UHPLC/MS/MS) optimized for basic species, UHPLC/MS/MS optimized for acidic species, and GC/MS (1, 6). Blood samples were collected in EDTA tubes and centrifuged at 3,000 rpm for 10 min at 4°C, with the plasma aliquoted, snap frozen in liquid nitrogen, and stored at -80°C until analysis. For each plasma sample, 100 μl was used for analyses. With the use of an automated liquid handler (Hamilton LabStar, Salt Lake City, UT), protein was precipitated from the plasma with methanol that contained four standards to report on extraction efficiency. The resulting supernatant was split into equal aliquots for analysis on the three platforms. Aliquots, dried under nitrogen and vacuum desiccated, were subsequently either reconstituted in 50 μl 0.1% formic acid in water (acidic conditions) or in 50 μl 6.5 mM ammonium bicarbonate in water, pH 8 (basic conditions) for the two UHPLC/MS/MS analyses or derivatized to a final volume of 50 μl for GC/MS analysis using equal parts bistrimethyl-silyl-trifluoroacetamide and solvent mixture acetonitrile-dichloromethane-cyclohexane (5:4:1) with 5% triethylamine at 60°C for 1 h. In addition, three types of controls were analyzed in concert with the experimental samples: aliquots of a well-characterized human plasma pool served as technical replicates throughout the data set, extracted water samples served as process blanks, and a cocktail of standards spiked into every analyzed sample allowed instrument performance monitoring. Standards to monitor extraction were d6-cholesterol, fl and tridecanoic acid. A standard to monitor GC/MS derivatization was 2-tert-butyl-6-methylphenol (BHT). GC/MS standards to monitor GC and MS performance were C5-C18 alkylbenzenes. Experimental samples and controls were randomized across platform run days. Instrument variability was determined by calculating the median relative standard deviation (RSD) for the internal standards that were added to each sample before injection into the mass spectrometers. Overall process variability was determined by calculating the median RSD for all endogenous metabolites (i.e., noninstrument standards) present in technical replicates of MTRX3. Values for instrument (5%) and process variability (12%) met Metabolon's acceptance criteria.

For UHLC/MS/MS analysis, aliquots were separated using a Waters Acquity UPLC (Waters, Millford, MA) instrument with separate acid/base-dedicated 2.1 mm X 100 mm Waters BEH C18 1.7-μm particle columns heated to 40°C, and analyzed using an LTQ mass spectrometer (Thermo Fisher Scientific, Waltham, MA), which consisted of an electrospray ionization (ESI) source and linear ion-trap (LIT) mass analyzer (6). Extracts reconstituted in formic acid were gradient eluted at 350 μl/min using 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B) (0% B to 70% B in 4 min, 70–98% B in 0.5 min, 98% B for 0.9 min), whereas extracts reconstituted in ammonium bicarbonate used 6.5 mM ammonium bicarbonate in

Table 1. *Subject characteristics*

Variable	Means ± SE
Age, yr	38.0 ± 1.6
Height, m	1.81 ± 0.02
Weight, kg	76.8 ± 2.3
Body fat, %	14.0 ± 1.0
$Watts_{max}$	304 ± 10.5
$\dot{V}O_{2max}$, $ml \cdot kg^{-1} \cdot min^{-1}$	51.7 ± 1.4
HR_{max}, beats/min	179 ± 2.5
Training, km/wk	192 ± 18.0

n = 19 subjects; $\dot{V}O_{2max}$, maximal oxygen consumed; HR, heart rate.

Table 2. *Average 75-km metabolic and performance data*

Variable	Means ± SE
Time, h	2.71 ± 0.07
V_{O_2}, ml·kg^{-1}·min^{-1}	38.3 ± 1.2
V_{O_2},%$V_{O_{2max}}$	69.3 ± 2.1
Watts	207 ± 7.6
%Watts$_{max}$	68.3 ± 1.7
HR, beats/min	150 ± 2.5
%HR$_{max}$	84.0 ± 1.1
Ventilation, l/min	74.9 ± 3.5
RPE	12.9 ± 0.3

V_{O_2}, volume of oxygen consumed; RPE, rating of perceived exertion.

water, pH 8 (A) and 6.5 mM ammonium bicarbonate in 95/5 methanol-water (B) (same gradient profile as above) at 350 μl/min. The MS instrument scanned 99–1,000 molecular weight (*m/z*) and alternated between MS and MS2 scans using dynamic exclusion with approximately 6 scans per second. Derivatized samples for GC/MS were separated on a 5% diphenyl-95% dimethyl polysiloxane-fused silica column with helium as the carrier gas and a temperature ramp from 60°C to 340°C and then analyzed on a Thermo-Finnigan Trace DSQ MS (Thermo Fisher Scientific) operated at unit mass resolving power with electron impact ionization and a 50- to 750-atomic mass unit scan range. Metabolites were identified by automated comparison of the ion features in the experimental samples to a reference library of chemical standard entries that included retention time, molecular weight (*m/z*), preferred adducts, and in-source fragments as well as associated MS spectra, and were curated by visual inspection for quality control using software developed at Metabolon (Durham, NC) (4). Common and biologically abundant isomers of unsaturated fatty acids containing the n3, n6, and n9 configuration are contained in Metabolon's chemical standard library. Liquid chromatography mass spectrometry (LC-MS) standards to monitor LC and MS performance were d_3-leucine, chloro- and bromo-phenylalanine, d_2-maleic acid, amitriptyline, and d10-benzophenone (1). Biochemical identifications were based on three criteria: retention index within a narrow window of the proposed identification, accurate mass match to the library +/- 0.005 amu, and the MS/MS forward and reverse scores between the experimental data and authentic standards. The MS/MS scores were based on a comparison of the ions present in the experimental spectrum to the ions present in the library spectrum.

Statistical analysis. Data are expressed as means ± SE. For the metabolomics statistical analyses and data display purposes, any missing values were assumed to be below the limits of detection and these values were imputed with the compound minimum (minimum value imputation). Statistical analysis of log-transformed data was performed using "R" (R Foundation, from http://cran.r-project.org/), which is a freely available, open-source software package. One-way ANOVA with post hoc contrasts (*t*-tests) was performed to compare data across time points. An estimate of the false discovery rate (*q* value) was calculated to take into account the multiple comparisons that normally occur in metabolomic-based studies, with $q < 0.05$ used as an indication of high confidence in a result (34). Fold changes across time points were calculated using group averages of the median scaled intensity values. Plasma cytokines and F_2-IsoP were analyzed using one-way ANOVA with post hoc contrasts (*t*-tests) performed to compare data across time points. Correlations between 13-HODE + 9-HODE and other variables were made using Pearson's product-moment correlation coefficients.

RESULTS

Subjects included 19 competitive male cyclists (ages 27 to 49 yr) who successfully adhered to all aspects of the study design (see Table 1). Metabolic and performance data from the 75-km mountainous cycling time trial are summarized in Table 2. The cyclists were able to maintain a high power output (69% Watts$_{max}$) during the 75-km trial, with an average duration of 2.71 h.

The metabolomics analysis revealed 423 detectable compounds of known identity. After log transformation and imputation with minimum observed values for each compound, repeated measures ANOVA contrasts identified significant

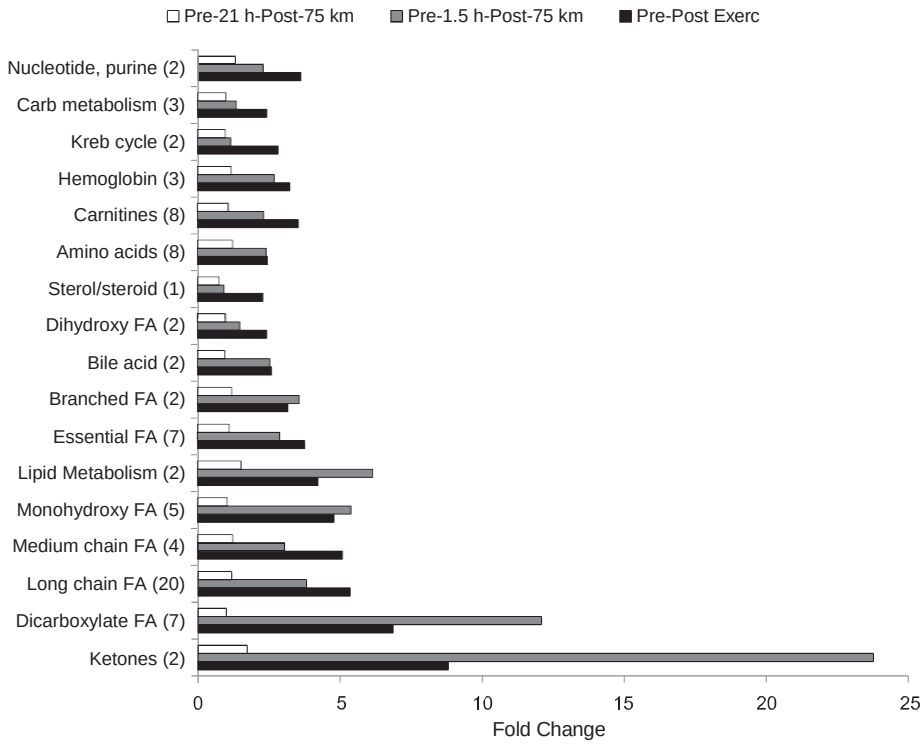

Fig. 1. Fold changes in metabolites grouped according to subpathways. Numbers in parentheses represent the number of metabolites in each subpathway that increased twofold or greater after the 75-km cycling time trial.

Table 3. *Exercise effects on linoleic acid and long chain polyunsaturated fatty acids in the conversion pathway (all time main effects, $q < 0.001$)*

Variable (Median Scaled Intensity)	Preexercise	Post-75 km	1.5-h Post-75 km	21-h Post-75 km
Linoleate (18:2n6)	0.76 ± 0.06	2.86 ± 0.15* (3.7-fold)	1.96 ± 0.11* (2.6-fold)	0.83 ± 0.05
Linolenate [ex or 'Y; (18:3n3 or 6)]	0.72 ± 0.07	4.23 ± 0.29* (5.8-fold)	2.43 ± 0.20* (3.4-fold)	0.76 ± 0.06
Dihomo-linolenate (20:3n3 or n6)	0.77 ± 0.04	2.66 ± 0.16* (3.4-fold)	2.18 ± 0.16* (2.8-fold)	0.84 ± 0.04
Arachidonate (20:4n6)	0.80 ± 0.06	1.83 ± 0.12* (2.3-fold)	1.55 ± 0.08* (1.9-fold)	0.84 ± 0.06
Adrenate (22:4n6)	0.62 ± 0.08	2.13 ± 0.12* (3.4-fold)	1.68 ± 0.17* (2.7-fold)	0.78 ± 0.11
Docosapentaenoate (22:5n6)	0.81 ± 0.06	1.59 ± 0.22* (2.0-fold)	1.87 ± 0.18* (2.3-fold)	0.94 ± 0.06

Values are means ± SE. *$P < 0.01$ vs. preexercise.

post-75 km cycling time effects for 221 metabolites. Fold changes were calculated from the median scaled intensity values for pre- to-post-75-km cycling time points and then rank ordered. A total of 80 metabolites of known identity had a twofold or higher increase following 75 km cycling (all q values <0.01). These were grouped by metabolic subpathways, and three mean fold changes were calculated (preexercise to immediately-, 1.5 h post-, and 21 h postexercise) (see Fig. 1 and Supplemental Table S1). All but 26 of these metabolites were related to lipid and carnitine metabolism, with the largest fold changes seen for ketones, dicarboxylate fatty acids, and long chain fatty acids.

Table 3 summarizes exercise-induced changes in median scaled intensity (MSI) values for linoleic fatty acid (18:2n6) and five other long chain polyunsaturated fatty acids in the conversion pathway. Fold changes for all of these fatty acids ranged from 2.0 to 5.8 immediately post-75 km cycling and 1.9 to 3.4 after 1.5 h recovery and were not different from preexercise after 21 h recovery. Plasma 13-HODE + 9-HODE increased 3.1- and 1.7-fold immediately and 1.5 h following the 75-km cycling time trial, respectively (time main effect, $q < 0.001$) (Fig. 2). Similar patterns of postexercise increases were seen for (*Z*)-9,10-dihydroxyoctadec-12-enoic acid (9,10-DiHOME) and (*Z*)-12,13-dihydroxyoctadec-9-enoic acid (12,13-DiHOME) (both time main effects, $q < 0.001$)(Fig. 3) and F_2-IsoP (time main effect, $P < 0.001$) (Fig. 4).

Plasma cytokine data are summarized in Table 4. Significant postexercise increases were measured for IL-6 (8.6-fold), IL-8 (2.3-fold), IL-10 (9.1-fold), TNF-ex (1.10-fold), MCP-1 (1.44-fold), and GCSF (1.38-fold), with values near preexercise levels after 21 h recovery (all time main effects, $P < 0.001$).

Immediate postexercise MSI values for 13-HODE + 9-HODE were significantly correlated with postexercise arachidonate ($r = 0.77$, $P < 0.001$), 12,13-DiHOME ($r = 0.60$, $P = 0.006$), and F_2-IsoP ($r = 0.75$, $P < 0.001$) (Fig. 5, *A–C*). Postexercise 13-HODE + 9-HODE was also significantly correlated with postexercise linoleate ($r = 0.54$, $P = 0.016$), dihomo-linolenate ($r = 0.57$, $P = 0.011$), and adrenate (r=0.56, $P = 0.013$), and marginally to linolenate ($r = 0.44$, $P = 0.058$) and 9,10-DiHOME ($r = 0.41$, $P = 0.081$). Postexercise levels of 13-HODE + 9-HODE were not correlated with any of the postexercise cytokine concentrations listed in Table 4. Immediate postexercise MSI values for linoleate were significantly correlated with 9,10-DiHOME ($r = 0.64$, $P = 0.003$), 12,13-DiHOME ($r = 0.58$, $P = 0.10$), and each fatty

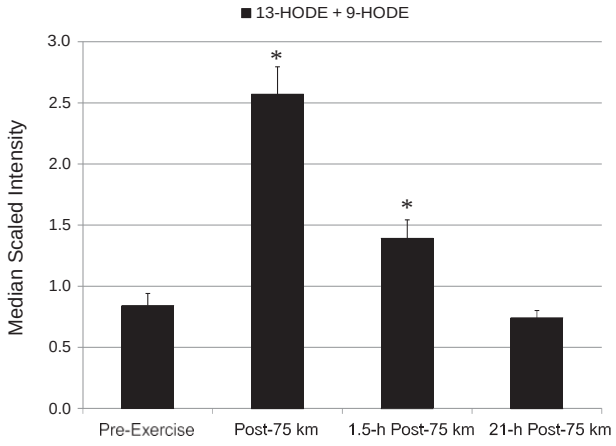

Fig. 2. Changes in 13- and 9-hydroxy-octadecadienoic acid (13-HODE + 9-HODE) across four time points: preexercise, immediately post-75-km cycling time trial, and 1.5-h and 21-h postexercise. *$P < 0.01$ compared with preexercise. Time main effect, $P < 0.001$.

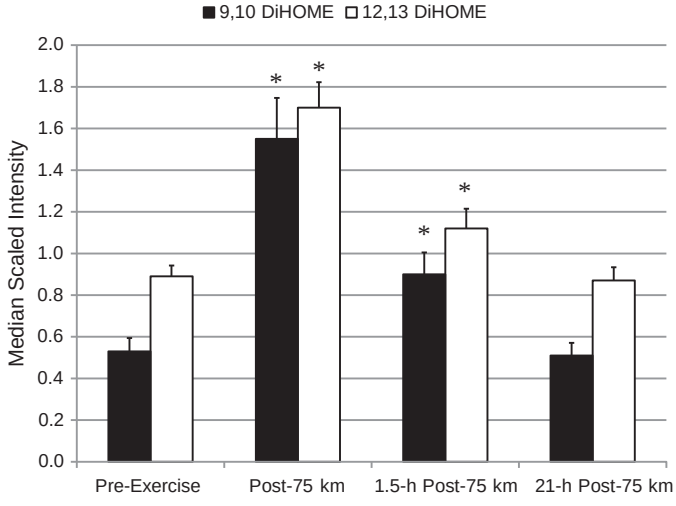

Fig. 3. Changes in (*Z*)-9,10-dihydroxyoctadec-12-enoic acid (9,10-DiHOME) and (*Z*)-12,13-dihydroxyoctadec-9-enoic acid (12,13-DiHOME) and across four time points: preexercise, immediately post-75-km cycling time trial, and 1.5-h and 21-h postexercise. *$P < 0.01$ compared with preexercise. Time main effect, $P < 0.001$.

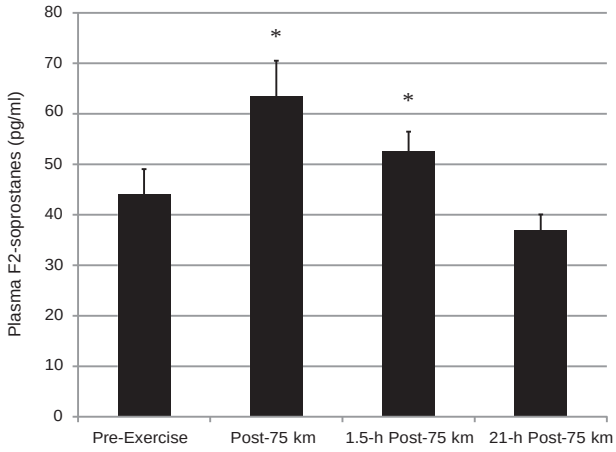

Fig. 4. Changes in F_2-isoprostanes across four time points: preexercise, immediately post-75-km cycling time trial, and 1.5-h and 21-h postexercise. *$P < 0.01$ compared with preexercise. Time main effect, $P < 0.001$.

acid listed in the conversion pathway except for docosapentaenote ($r = 0.32$, $P = 0.18$; all other relationships, $P < 0.02$).

DISCUSSION

Cyclists experienced a transient 3.1-fold increase in the oxidized linoleic acid derivative 13-HODE + 9-HODE following the 75-km cycling time trial. The postexercise increase in plasma concentration of HODEs was related to increases in the well-established oxidative stress biomarker, F_2-IsoP, but not to increases in inflammatory cytokines. Postexercise HODEs were also positively correlated with exercise-induced mobilization of most components of the linoleic acid conversion pathway including linoleate, dihomo-linolenate, arachidonate, and adrenate, and the oxidized isoleukotoxin 12,13-DiHOME.

Metabolomics data revealed a twofold or greater postexercise increase in 80 metabolites of known identity, and two-thirds were related to lipid and carnitine metabolism. These results are very similar to those of our previous metabolomics-based investigation of 15 runners who ran 2.5 h/day at 70% $\dot{V}O_{2max}$ 3 days in a row (20). Together, these two studies indicate that following prolonged and intensive exercise, endurance athletes experience profound systemic shifts in blood metabolites, especially those from the lipid pathway. In agreement with Lehman et al. (17), the data support substantial fatty acid transport across the mitochondrial membrane and oxidation during prolonged exercise.

Table 4. *Exercise effects on plasma cytokines (all time main effects, P < 0.001)*

Variable, pg/ml	Preexercise	Post-75 km	1.5-h Post-75 km	21-h Post-75 km
IL-6	1.02 ± 0.28	8.77 ± 0.69*	5.97 ± 0.97*	0.63 ± 0.05
IL-8	5.22 ± 0.42	11.83 ± 1.1*	7.91 ± 0.74*	4.70 ± 0.33
IL-10	2.05 ± 0.21	18.6 ± 2.6*	10.6 ± 1.8*	2.14 ± 0.27
TNF-ex	3.90 ± 0.16	4.30 ± 0.20*	4.10 ± 0.20	3.61 ± 0.16
MCP-1	267 ± 9.1	385 ± 18.9*	340 ± 22.5*	230 ± 7.1
GCSF	12.8 ± 1.0	17.7 ± 1.8*	18.7 ± 2.2*	15.3 ± 1.5

Values are means ± SE. IL, interleukin; TNF-ex, tumor necrosis factor-ex; MCP-1, monocyte chemoattractant protein-1; GCSF, granulocyte colony-stimulating factor. *$P < 0.01$ vs. preexercise.

Oleic (18:1), palmitic (16:0), and linoleic (18:2) are the three major free fatty acids (FFA) in adipose tissue (9, 15). Upper body subcutaneous adipose tissue is the primary source of FFA during prolonged and intensive exercise (11), and relative availability is the predominant determinant of individual FFA use by contracting muscles (13). Ketone bodies increased strongly in overnight fasted cyclists following the 75-km cycling time trial, indicating a high degree of FFA 13-oxidation.

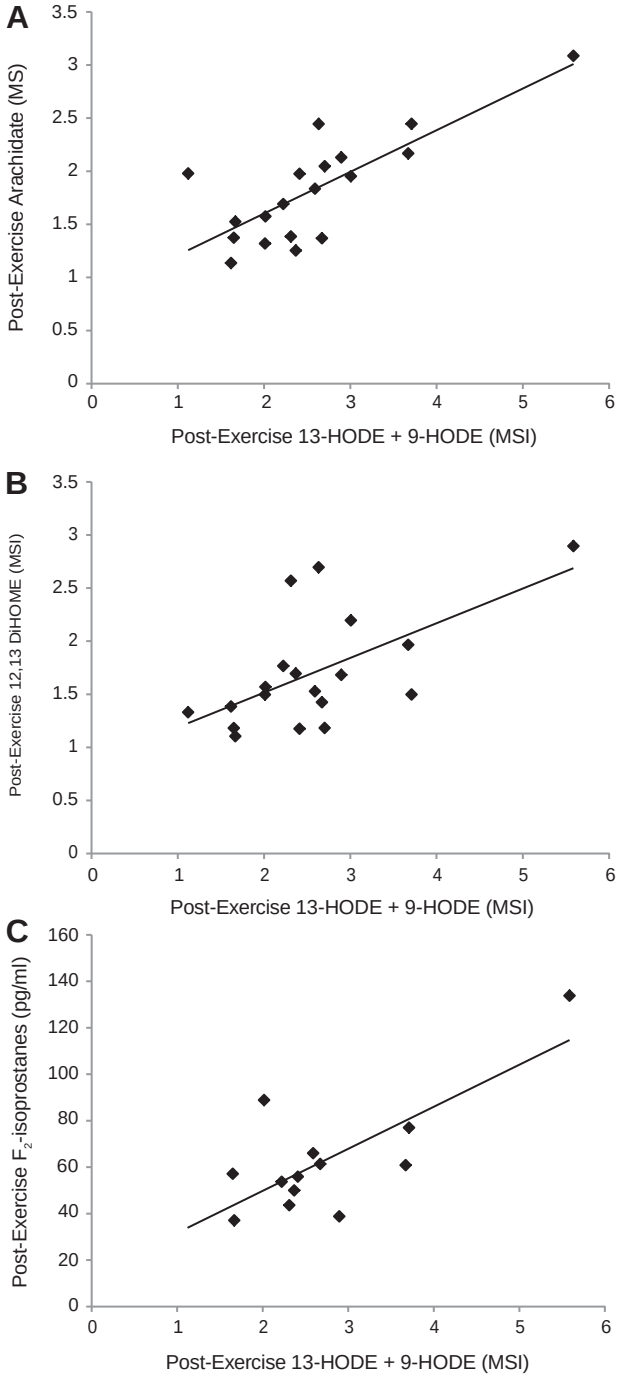

Fig. 5. Scatterplot relationships between at the postexercise time point for 13-HODE + 9-HODE and arachidonate ($r = 0.77$, $P < 0.001$) (*A*), 12,13-DiHOME ($r = 0.60$, $P = 0.006$) (*B*), and F_2-isoprostanes ($r = 0.75$, $P < 1.1$) (*C*). MSI, median scaled intensity.

Plasma linoleate increased 3.7-fold postexercise in concert with other fatty acids in the conversion pathway, indicating a high degree of mobilization and availability.

Linoleic acid is the predominant PUFA in the diet and adipose tissue stores and can be metabolized by cyclooxygenase, lipoxygenase, and P450 enzymes (32). 13-HODE + 9-HODE are monohydroxy lipoxygenation products and the most widely distributed of linoleic acid metabolites (17, 32). The HODEs are secreted by a variety of cells including macrophages, endothelial cells, platelets, and smooth muscle cells, and exert biological and signaling activities as PPAR and GPR132 ligands (12, 21, 28, 37). Cell injury activates lipoxygenases and may be one pathway through which intensive exercise increases production of HODES (33). Several cell culture studies indicate that HODES induce pro-inflammatory responses including generation of inflammatory cytokines such as IL-113 and IL-8 (16, 35), chemotactic activity of neutrophils (10), and stimulation of NF-KB activity (24). The data from this study, however, do not support that HODES are related to inflammatory cytokines within an exercise context despite similar patterns of change over time and peaking immediately postexercise.

Isoprostanes are prostaglandin-like compounds produced by the free radical catalyzed peroxidation of arachidonic acid independent of cyclooxygenase (22, 30). A specific class of isoprostanes, the F_2-IsoP, are an accurate indicator of in vivo oxidant stress and have been related to a variety of human disorders (30). The tight linkage between postexercise levels of 13-HODE + 9-HODE and F_2-IsoP is a novel finding from this study. Plasma concentrations of F_2-IsoP are much lower than 13-HODE + 9-HODE (39). Linoleate is the most abundant fatty acid in atherosclerotic plaques, and high levels of HODEs accumulate in the low-density lipoprotein (LDL) and plaque of patients with atherosclerosis compared with healthy controls (14). Similar to F_2-IsoP, the HODEs are stable lipid peroxidation products that are elevated in diseased individuals and can be lowered through positive lifestyle changes (2, 3, 7, 25, 29, 37). The data from this study support the use of 13-HODE + 9-HODE as biomarkers of oxidative stress during exercise trials.

Postexercise 13-HODE + 9-HODE was significantly correlated with 12,13-DiHOME, another derivative of linoleic acid diol. 12,13-DiHOME is produced via the oxidation of the isoleukotoxin 12,13-epoxyoctadecenoic acid (12,13-EpOME). Both 12,13-DiHOME and 12,13-EpOME are PPAR-'Y ligands with potentially wide-ranging effects. In addition to its role as a PPAR ligand, 12,13-DiHOME exerts toxic and oxidative effects, inhibits mitochondrial function, stimulates neutrophil chemotactic activity, and suppresses neutrophil respiratory burst activity (5, 31, 36, 38).

Perspectives and Significance

We recently reported that a 3-day period of intensified training elicited large changes in the human serum metabolome of runners (20). Athletes ran for 2.5 h/day on treadmills at ~70% $\dot{V}O_{2max}$ for 3 days in a row, with blood samples collected preexercise and immediately and 14 h postexercise. Immediately after the 3-day exercise period, significant twofold or higher increases in 75 metabolites were measured, with all but 22 of these metabolites related to lipid/carnitine metabolism. We reported a fivefold postexercise increase in plasma 13-HODE + 9-HODE, with levels returning to preexercise levels within 14-h recovery (20). Although this was the first published information on the acute increase in plasma 13-HODE + 9-HODE following intensive exercise, other investigators using disease models had reported that higher HODE concentrations were excellent indicators of oxidative stress and should be considered in future studies because they were much more abundant than F_2-isoprostanes (37, 39). A major objective in the current study was to determine whether 13-HODE + 9-HODE could be related to F_2-isoprostanes within an exercise context, and this was established. In this study, cyclists completing a 75-km time trial experienced twofold or higher increases in 80 metabolites, including linoleate and other fatty acids in the conversion pathway and the oxidized derivatives 13-HODE + 9-HODE. Although 13-HODE + 9-HODE play proinflammatory roles under certain conditions, no relationship to postexercise increases in six cytokines was established in this study. The 3.1-fold postexercise increase in HODEs was significantly correlated with F_2-IsoP, supporting the inclusion of HODEs as a stable oxidative stress biomarker in future exercise trials.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: D.C.N., R.A.S., and M.P.M. conception and design of research; D.C.N., R.A.S., B.L., M.P.M., D.A.D., and K.L.P. performed experiments; D.C.N. and K.L.P. analyzed data; D.C.N., R.A.S., B.L., M.P.M., D.A.D., and K.L.P. interpreted results of experiments; D.C.N. and K.L.P. prepared figures; D.C.N. drafted manuscript; D.C.N., B.L., M.P.M., D.A.D., and K.L.P. edited and revised manuscript; D.C.N., R.A.S., B.L., M.P.M., D.A.D., and K.L.P. approved final version of manuscript.

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