Metabolic and hormonal responses to isoenergetic high-intensity interval exercise and continuous moderate-intensity exercise

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This study investigated the effects of high-intensity interval training (HIIT) vs. work-matched moderate-intensity continuous exercise (MOD) on metabolism and counterregulatory stress hormones. In a randomized and counterbalanced order, 10 well-trained male cyclists and triathletes completed a HIIT session [81.6 ± 3.7% maximum oxygen consumption (VO2 max): 72.0 ± 2.2% peak power output; 792 ± 95 kJ] and a MOD session (66.7 ± 3.5% VO2 max: 48.5 ± 3.1% peak power output; 797 ± 95 kJ). Blood samples were collected before, immediately after, and 1 and 2 h postexercise. Carbohydrate oxidation was higher (P = 0.037; 20%), whereas fat oxidation was lower (P = 0.037; −47%) during HIIT vs. MOD. Immediately after exercise, plasma glucose (P = 0.024; 20%) and lactate (P < 0.01; 5.4%) were higher in HIIT vs. MOD, whereas total serum free fatty acid concentration was not significantly different (P = 0.33). Targeted gas chromatography-mass spectrometry metabolomics analysis identified and quantified 49 metabolites in plasma, among which 11 changed after both HIIT and MOD, 13 changed only after HIIT, and 5 changed only after MOD. Notable changes included substantial increases in tricarboxylic acid intermediates and monounsaturated fatty acids after HIIT and marked decreases in amino acids during recovery from both trials. Plasma adrenocorticotropic hormone (P = 0.019), cortisol (P < 0.01), and growth hormone (P < 0.01) were all higher immediately after HIIT. Plasma norepinephrine (P = 0.11) and interleukin-6 (P = 0.20) immediately after exercise were not significantly different between trials. Plasma insulin decreased during recovery from both HIIT and MOD (P < 0.01). These data indicate distinct differences in specific metabolites and counterregulatory hormones following HIIT vs. MOD and highlight the value of targeted metabolomic analysis to provide more detailed insights into the metabolic demands of exercise.

As an extension to this early research on intensity-dependent metabolic changes, more recent research has compared molecular adaptations to high-intensity interval exercise and continuous, moderate-intensity exercise. Most research in this area has focused on short intervals of 30 s; however, several other studies have investigated the effects of longer intervals lasting 2–4 min (2, 21, 57, 65). Markers of mitochondrial biogenesis increase to a similar extent after these forms of exercise, both acutely (2, 17) and chronically (7). Few studies have compared differences in CHO, fat, and amino acid metabolism between high-intensity intervals and continuous, moderate-intensity exercise (2). Furthermore, other studies comparing the effects of high-intensity intervals and continuous, moderate-intensity exercise on long-term changes in blood lipids and body fat percentage have reported variable findings (45, 56). Considering the potential benefits of HIIT (19), further research to compare differences in metabolism between this form of training and more traditional continuous, moderate-intensity exercise is warranted.

Metabolomics is a relatively new analytical platform within the domain of exercise biochemistry and physiology. Coupled with the greater availability and ease-of-use of mass-spectrometry and nuclear magnetic resonance equipment, we now have better access to powerful data acquisition and advanced data-processing techniques to identify large numbers of analytes simultaneously in biological specimens (48). Metabolomics and proteomics were first applied to exercise studies on animals (5) and then humans (51). These early studies helped to refine these techniques for use in exercise research but did not...
yield any detailed information on specific metabolic responses to exercise (51). Subsequently, metabolomics and proteomics have been used to determine responses to acute exercise (34, 42, 43, 48) and in response to exercise training (49, 67). However, we are not aware of any studies that have systematically employed metabolomics to compare metabolic responses to different modes of exercise.

CHO and fat metabolism during exercise is regulated by various hormones, including insulin, glucagon, cortisol, adrenocorticotrophic hormone (ACTH), growth hormone, epinephrine, and norepinephrine (37). The cytokine interleukin (IL)-6 also influences metabolism during exercise in a hormone-like manner (50). Combining metabolomics with a range of counterregulatory hormones provides a more complete perspective on cross talk between the endocrine system and metabolic organs such as skeletal muscle, adipose tissue, and the liver during exercise.

The primary aim of this study was to compare the metabolic responses after high-intensity interval and continuous, moderate-intensity cycling matched for total workload. To achieve this goal, we employed targeted gas chromatography–mass spectrometry (GC-MS)-based metabolic analysis to assess changes in tricarboxylic acid (TCA) intermediates, fatty acids, and amino acids. A secondary aim was to investigate changes in counterregulatory hormones that regulate metabolism during exercise and are responsive to exercise intensity and duration (e.g., ACTH hormone, cortisol, catecholamines, growth hormone, glucagon, IL-6, and insulin). We hypothesized that CHO and TCA metabolism would be greater, whereas fat metabolism would be lower, after high-intensity interval cycling compared with continuous, moderate-intensity cycling.

METHODS

Participants. Ten well-trained male cyclists and triathletes volunteered to participate in the study. The characteristics of these athletes were as follows: mean ± SD age 33.2 ± 6.7 yr; height 1.83 ± 0.06 m; body mass 78.6 ± 8.7 kg; body mass index 23.4 ± 2.7 kg/m²; VO_{2max} 4.8 ± 0.3 l/min; peak power output 443 ± 37 W; and maximum heart rate (HR) 188 ± 11 beats/min. Inclusion criteria for this study required: 1) one year competitive cycling or triathlon experience, 2) high-intensity training in the 2 mo before participation, and 3) good physical health as indicated by a medical screening questionnaire. Written informed consent was obtained before study participation, and ethical clearance was obtained from the Human Research Ethics Committee at The University of Queensland.

Preliminary testing. The athletes were required to visit an exercise laboratory in the School of Human Movement Studies at The University of Queensland on three occasions before the experimental trials. On the first two occasions, the athletes were tested to determine their VO_{2max}. On the third occasion, the athletes completed a familiarization trial for the HIIT.

VO_{2max} and peak power output. VO_{2max} was determined before the experimental trials using a graded exercise test to volitional fatigue on an electromagnetically braked cycle ergometer (Lode Excalibur; Lode BV, Groningen, The Netherlands). Following 5–10 min warm-up at a self-selected intensity, the test began at 100 W, and the power increased by 15 W increments every 30 s until volitional exhaustion. Athletes were required to maintain their cadence above 60 revolutions/min (rpm). HR was recorded continuously during the test using a radiotelemetry HR monitor (Polar Electro; Oy, Kempele, Finland). Peak power output was established either as 1) the power output associated with the final completed stage of the test or 2) by multiplying the fraction of time spent in the final incomplete stage by 15 W and adding this to the power output in the prior completed stage of the test. The test was repeated at least 48 h later, and the higher VO_{2max} and associated peak power output were used to determine power output during the experimental trials.

Familiarization trial. To determine whether the athletes could complete the HIIT session, they completed a familiarization trial at least 48 h following the second VO_{2max} test. This required them to complete 10 × 4 min intervals at a power output corresponding to ~80% VO_{2max} with a 2-min recovery at 50 W (11.4 ± 0.9% peak power output) between the intervals. The work completed during each interval was calculated by multiplying the power output for each athlete at 80% VO_{2max} by the duration of the interval (240 s). The work completed during each recovery period was calculated by multiplying the power output (50 W) by the duration of the recovery period (120 s). These two figures were added together and then multiplied by 10 to calculate the total amount of work that each athlete completed in this familiarization trial. This figure was then used to determine the duration of exercise required to complete the same amount of work while cycling continuously at the power output corresponding to ~65% VO_{2max} (moderate-intensity continuous exercise, MOD). The required duration was calculated by dividing the total amount of work that each athlete completed in this familiarization trial by the power output for each athlete at 65% VO_{2max}.

Exercise trials. At least 1 wk after the familiarization trial the athletes completed either the HIIT or MOD trial in a randomized and counterbalanced order. These two trials were separated by a minimum of 7 days. On the morning of each trial, the athletes arrived at the laboratory around 6:30 A.M. Upon arrival, a preexercise venous blood sample (20 ml) was collected from an antecubital vein. The athletes then proceeded with a 10- to 15-min warm-up at a self-selected intensity before each experimental trial. The intensity and duration of warm-up were recorded for replication in the second trial. HR and ratings of perceived exertion at the end of warm-up were also noted. HR, ratings of perceived exertion, VO_{2}, and VCO_{2} were recorded continuously from 0- to 4-min periods at four different time points during both trials (i.e., 0–4, 18–22, 36–40, and 48–52 min). The data-logged VO_{2} and VCO_{2} were averaged over 4 min, and these averages were then used to calculate rates of substrate oxidation (see details below). Immediately after each period of gas analysis, ear prick blood samples were collected and analyzed to measure capillary lactate concentration using a Lactate Pro kit (Arkray Factory, Shiga, Japan). Within 5 min of the end of exercise, another venous blood sample was collected. Additional venous blood samples were collected 1 and 2 h after exercise while the athletes rested quietly and consumed water ad libitum.

Dietary and exercise training control. The athletes were requested to avoid alcohol and caffeine and consume similar foods 24 h before both experimental trials with the assistance of a food diary. In addition they were provided with a standardized preexercise meal consisting of six Sanitarium Weet-bix and sufficient Sustagen Sport powder mixed with milk to provide each athlete with 1.5 g CHO/kg body mass. The athletes consumed this meal 1.5 h before arrival at the laboratory for both experimental trials. They also completed training diaries and were requested to follow the same training schedule in the week before each experimental trial. In the 24 h before each trial, participants were reminded to keep physical activity to a minimum and avoid intense training.

Substrate oxidation. Rates of total CHO and fat oxidation were calculated using stoichiometric equations (25), with the assumption that protein oxidation was negligible:

\[ \text{CHO oxidation (g/min)} = 4.210 \cdot \dot{V}_{\text{CO}_2} - 2.962 \cdot \dot{V}_{\text{O}_2} \]

\[ \text{Fat oxidation (g/min)} = 1.695 \cdot \dot{V}_{\text{O}_2} - 1.701 \cdot \dot{V}_{\text{CO}_2} \]

The rate of energy expenditure was calculated using the following formula, where \(\dot{V}_{\text{O}_2}\) and \(\dot{V}_{\text{CO}_2}\) are in l/min.
Energy expenditure (kJ/min) = 16.318 · VO₂ + 4.602 · VCO₂.

Blood sampling. At each of the four time points (preexercise, postexercise, 1 and 2 h postexercise), blood samples were collected in four 6-ml Vacutainers. Three Vacutainers contained anticoagulants (EDTA, lithium heparin, or fluoride/oxalate). EDTA, lithium heparin, and fluoride/oxalate samples were placed on ice, whereas serum sample collected in a serum separation tube was left to clot at room temperature for 20–30 min. Once the serum sample had clotted, all samples were centrifuged at 2,500 rpm for 10 min at 4°C. Plasma and serum samples were separated into aliquots and stored in Eppendorf tubes at −80°C until further processing. One aliquot of lithium heparin plasma was mixed with 11 μl 5.26 mmol/l sodium metabisulfite before freezing to prevent oxidation of catecholamines.

Blood analysis. Enzymatic assay kits were used to measure glucose and lactate (bioMérieux) in fluoride/oxalate plasma and total nonesterified free fatty acids (Wako Diagnostics, Richmond, VA) in serum. These assays were performed in duplicate on an automated biochemistry analyzer (Cobas Mira; Roche Diagnostics). The intra-assay coefficient of variation was 2.0% for glucose, 2.2% for lactate, and 2.6% for nonesterified free fatty acids. ACTH, cortisol, growth hormone, and insulin concentrations were measured in duplicate using EDTA plasma and immunoassay kits customized on an automated analyzer (Cobas e411; Roche Diagnostics, Mannheim, Germany). The intra-assay coefficient of variation was 8.3% for ACTH, 2.2% for cortisol, 2.3% for growth hormone, and 4.6% for insulin. Cortisol-binding globulin was measured in duplicate by ELISA (Cusabio, Hubei, China). Free cortisol was then calculated and reported as the ratio between cortisol and cortisol-binding globulin (33). IL-6 and glucagon were also measured in duplicate by ELISA (R&D Systems Quantikine). The intra-assay coefficient of variation was 6.0% for cortisol-binding globulin, 6.4% for IL-6, and 7.8% for glucagon. Epinephrine and norepinephrine were measured in single by HPLC. The intra-assay coefficient of variation was 6.5% for norepinephrine and 5.3% for epinephrine.

Metabolomics analysis. Lithium heparin plasma samples were thawed, and 300 μl were separated into aliquots with the addition of 20 μl of 10 mmol/l DL-alanine-2,3,3,3-d₄ (D₄-alanine; Sigma–Aldrich) as an internal standard. All samples were vortexed for 1 min and frozen at −80°C. The samples were then lyophilized overnight using an SC250 Express SpeedVac Concentrator and RV74104 Refrigerated Vapor Trap (Thermo Scientific Savant). Dried pellets were resuspended in 500 μl of 50% methanol and vortexed vigorously for 1 min. The suspensions were centrifuged for 5 min at 4°C at 3,500 rpm. The supernatants were collected, transferred into a 15-ml falcon tube, and kept on dry ice. The pellets were resuspended in 500 μl of 80% methanol, and the suspensions were recentrifuged under the same conditions mentioned above. After the supernatants were pooled from the two centrifuge steps, the volume of supernatant was increased to 5–6 ml using milli-Q water, vortexed, and frozen overnight at −80°C. Extracted frozen supernatants were further lyophilized overnight as described previously. All tubes were then stored at −80°C until the derivatization step.

Methyl chloroformate derivatization was based on an optimized protocol reported previously (53). Lyophilized samples were resuspended in 1 mol/l sodium hydroxide (200 μl) and vigorously vortexed. After the resuspended samples were transferred to silanized tubes, methanol (334 μl) and pyridine (67 μl) were added. To initiate the derivatization process, methylichloroformate (40 μl) was added followed by vigorous mixing for 30 s. After this step was repeated, chloroform (400 μl) was added, and the mixture was vortexed for 10 s to separate the methylichloroformate derivatives from the reaction mixture. Sodium bicarbonate (400 μl, 50 mmol/l) was then added, and the mixture was vortexed for 10 s. Thereafter, samples were centrifuged for 5 min at 5°C at 2,000 rpm to separate the aqueous layer from the organic layer. With the use of a glass Pasteur pipette and bung, the upper aqueous layer was removed, and anhydrous sodium sulfate (100–150 mg) was added to remove any remaining water. Once dried, the remaining chloroform solution was transferred to a GC-MS vial assembled with a glass insert and airtight lid.

GC-MS instrument parameters were set according to the protocol described previously (53). Analysis was performed using an Agilent 7890A gas chromatograph coupled to an MSD-5975C inert mass spectrometer (Agilent). A split/splitless inlet was used for the identification of metabolites. With the use of a CTC PAL autosampler (CTC Analytics), 1 μl of sample was injected into a glass split/splitless 4-mm-inner diameter (ID) straight inlet liner packed with deactivated glass wool (Supelco). The inlet was set to 290°C and 56.8 kPa. It was then pulsed splitless at 180 kPa for 1 min with column flow of 1 ml/min. Together, an average initial linear velocity of 35 cm/s was achieved. Approximately 1 min after the injection, the purge flow was set to 25 ml/min.

A ZB1701 (Zebtron) capillary column [30 m × 250 μm (ID) × 0.15 μm film thickness] (Phenomenex) was used for all measurements. Carrier gas was ultra-high-purity grade helium (99.99%; BOC). GC oven temperature programming started isothermally at 45°C for 2 min. It was then increased at 9°C/min to 180°C and maintained for 5 min, increased by 40°C/min to 220°C and maintained for 5 min, increased by 40°C/min to 240°C and maintained for 11.5 min, and finally increased by 40°C/min to 280°C and maintained for 2 min. The transfer line to the mass selective detector was maintained at 250°C, the source at 230°C and the quadrupole at 150°C. At 5.5 min into the run, the detector was turned on and operated in positive-ion, electron-impact ionization mode at 70 eV electron energy with the electron multiplier set with no additional voltage relative to the autotune value. To monitor instrumental carryover, N-hexane blanks (Merck) were run after every 10 samples. Identification of species was carried out using mass spectra acquired in scan mode from 38 to 550 amu, with detection threshold of 100 ion counts. The raw data obtained from GC-MS was analyzed using the R package Metab and Automated Mass Spectral Deconvolution and Identification System. The obtained data were corrected for internal standard (D₄-alanine) and volume. Data for metabolomic species are reported as internal standard-normalized peak heights.

Statistical analysis. Data were analyzed using SPSS version 18.0 (Chicago, IL). Normality of the distribution for outcome measures was tested using the Shapiro–Wilk test. When necessary, raw data were log-transformed to obtain normality. Normally distributed data were analyzed using 2 (trial) × 4 (time) factor repeated-measures analysis of variance. If this analysis revealed any significant time and time × trial interaction effects (P < 0.05), paired t-tests were used to compare changes over time within trials, and differences between trials. Data that were not normally distributed after log transformation were analyzed using the nonparametric Friedman’s test. If the result of this test was significant (P < 0.05), Wilcoxon’s signed-rank tests were used to compare changes within trials, and differences between trials. The false discovery rate was used to adjust P values for multiple comparisons (8). Respective P values at or below critical values of 0.05, 0.033, and 0.016 for changes within trials and P values at or below critical values of 0.05, 0.038, 0.025, and 0.013 for difference between trials were accepted as statistically significant. Data that did not require log transformation are reported as means ± SD, whereas log-transformed data are reported as geometric means ± 95% confidence interval. Data that were not normally distributed are reported as median, 25th, and 75th percentiles.

Data processing. The metabolomic compound names were entered into the Human Metabolome Database version 3.5 (http://www.hmdb.ca/) to retrieve KEGG compound identifiers. This list of identifiers was then used to search the KEGG BRITE compound classification hierarchy (http://www.genome.jp/kegg/tool/map_brite1.html). This hierarchical information was used to create a binary network of classification terms and species to import into Cytoscape (http://www.cytoscape.org/) to generate a network map. Statistical analysis
including time and interaction effects and fold changes for each metabolomic compound were then imported as a separate network attributes file and used to specify visual properties on the network. In addition to quantitative statistical analysis, a heat map depiction of the percentage change in each of the plasma metabolites from preexercise levels was generated for the entire metabolomics dataset using the gplots package within the R statistical software (heat map.2 function) to display relative changes over time in families of metabolites in response to exercise trials.

RESULTS

Exercise intensity and substrate oxidation. The duration of exercise was exactly 60 min for the HIIT trial and 61 min ± 14 s for the MOD trial. The total amount of work completed during exercise was not significantly different between the HIIT and MOD trials (P = 0.71) (Table 1). HR, \( \dot{\text{V}}\text{O}_{2} \), %\( \dot{\text{V}}\text{O}_{2\text{max}} \), power output, %peak power output, and the rate of energy expenditure were all higher during HIIT compared with the corresponding periods during MOD (P < 0.05) (Table 1). The rates of CHO oxidation (Table 1) and blood lactate concentration (Fig. 1) were also higher during HIIT compared with MOD (P < 0.01). Conversely, the rate of fat oxidation tended to be lower (P = 0.037), whereas respiratory exchange ratio tended to be higher (P = 0.063), during HIIT compared with MOD (Table 1).

Glucose, lactate, and free fatty acids. Blood lactate was higher throughout HIIT compared with MOD (P < 0.01; Fig. 1A). Compared with preexercise values, plasma glucose concentration (P < 0.01; Fig. 1B) and total serum free fatty acid concentration (P < 0.001; Fig. 1C) increased after both HIIT and MOD. Compared with MOD, both plasma glucose and lactate (Fig. 1D) were higher immediately after HIIT (P < 0.05). Plasma lactate concentration increased after HIIT (P < 0.01) but not MOD (P = 0.29) (Fig. 1). Total serum free fatty acid concentration was not significantly different between the trials (P = 0.33). During the postexercise recovery period, plasma glucose concentration returned to preexercise values within 1 h after both trials. Plasma lactate concentration remained higher 1 h after HIIT compared with MOD (P < 0.05). Lactate was below preexercise values 2 h after HIIT (P < 0.01). Serum free fatty acid concentration remained higher at 1 and 2 h after both HIIT and MOD (P < 0.01).

Metabolomic compounds. Targeted metabolomic analysis identified 49 compounds in the plasma samples. According to KEGG classification, these species were categorized into various types of metabolites, including fatty acids (saturated,
monounsaturated, polyunsaturated), amino acids (branched chain, essential, nonessential), carboxylic acids, fatty acyl carnitines, and unclustered species. The network diagram in Fig. 2A summarizes the distribution of these metabolites, and significant changes observed over time. Among the 49 metabolites that were identified, 29 changed significantly after exercise ($P < 0.05$); among these 29 metabolites, 11 changed after both HIIT and MOD, 13 changed only after HIIT, and 5 changed only after MOD (Fig. 2, B and C). The heat map in Fig. 3 summarizes the relative magnitude of changes in each of the detected metabolites from preexercise levels in response to HIIT and MOD.

The TCA cycle intermediates citric acid (Fig. 4A), succinic acid (Fig. 4B), aconitic acid (Fig. 4C), and malonic acid (Fig. 4D) all increased after HIIT ($P < 0.01$). Furthermore, citric acid, succinic acid, and aconitic acid were all higher after HIIT compared with MOD ($P < 0.01$). Other carboxylic acids such as 4-methyl-2-oxopentanoic acid and 3-methyl-2-oxopentanoic acid also increased after HIIT ($P < 0.01$) (data not shown). Only succinic acid increased significantly after MOD ($P < 0.01$).

Most amino acids did not change during HIIT or MOD, with the exception of the nonessential amino acids alanine (Fig. 5A), glutamate (Fig. 5B), and tyrosine (data not shown), which increased following HIIT ($P < 0.05$). Alanine was also higher immediately after HIIT compared with MOD ($P < 0.01$). During recovery from exercise, the branched-chain amino acids leucine (Fig. 5C), valine (Fig. 5D), and isoleucine (data not shown); the essential amino acid methionine (Fig. 5E); and the nonessential amino acids alanine (Fig. 5A) and proline (Fig. 5F) all decreased below preexercise values, particularly after MOD ($P < 0.01$).

**Fig. 2.** Network map (A) and KEGG classification of metabolomic species (B) identified and quantified in plasma by gas chromatography-mass spectrometry. Numbered nodes represent measured metabolites. Nodes with colored letters represent a significant change with time for specific species after HIIT (H) and MOD (M) exercise. Numbered nodes with red boarders represent those metabolites that had a significantly different response between high-intensity intervals and continuous, moderate-intensity exercise. The Venn diagram (C) illustrates the distribution of common and trial-specific changes in metabolomic species after exercise.
Fig. 3. Heat map showing fold changes in metabolomic compounds after high-intensity intervals and continuous, moderate-intensity exercise. PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids. Numbers on the right correspond to compound numbers in Fig. 2 and KEGG compound identifiers.
The saturated fatty acids myristic acid (Fig. 6A), dodecanoic acid (Fig. 6B), and decanoic acid (Fig. 6C) all increased after both trials \((P < 0.01)\). The monounsaturated fatty acids palmitoleic acid (Fig. 6D) and heptadecenoic acid (data not shown) increased after both trials \((P < 0.05)\), whereas myristoleic acid (Fig. 6E) and oleic acid (Fig. 6F) increased only after HIIT \((P < 0.05)\) (Fig. 6). The polyunsaturated fatty acid \(\gamma\)-linolenic acid increased after MOD \((P = 0.005)\), and there was a trend toward an increase after HIIT \((P = 0.037)\) (data not shown). The increase in some of these fatty acids (e.g., oleic acid, myristoleic acid) after exercise was relatively brief, whereas other fatty acids (e.g., myristic acid, palmitoleic acid) remained elevated during the 2-h recovery period.

The percentage molar distribution of all nonesterified fatty acid (NEFA) detected in plasma was calculated using normalized peak heights as previously described (39) and is presented in Table 2. Among the fatty acids that increased after exercise, oleic acid was the most abundant fatty acid, whereas myristoleic acid was the least abundant. The molar distribution of dodecanoic acid, myristic acid, and palmitoleic acid increased after both HIIT and MOD \((P < 0.05)\), the molar distribution of octanoic acid decreased after MOD \((P < 0.05)\), and the molar distribution of linoleic acid decreased after HIIT \((P < 0.05)\).

Other metabolomic species that were present in plasma (including glycine, phenylalanine, cysteine, threonine, ornithine, arachidonic acid, adrenic acid, docosahexaenoic acid, eicosapentaenoic acid, bis-homo-\(\gamma\)-linolenic acid, azelaic acid, octanoyleacetic acid, palmitic acid, stearic acid, arachidic acid, quinic acid, heptadecanoic acid, phenylacetic acid) did not change significantly after exercise \((P > 0.05)\).

**Hormones.** The plasma concentrations of growth hormone (Fig. 7A), norepinephrine (Fig. 7B), and IL-6 (Fig. 7C) increased after both HIIT and MOD, whereas ACTH (Fig. 7D), free cortisol (Fig. 7E), and epinephrine (Fig. 7F) increased only after HIIT \((P < 0.05)\) (Fig. 7). Compared with MOD, the plasma concentrations of ACTH, free cortisol, and growth hormone were all higher immediately after HIIT \((P < 0.05)\). During the postexercise recovery period, growth hormone concentration remained elevated up to 2 h after MOD and up to 2 h after HIIT \((P < 0.05)\), whereas IL-6 remained elevated up to 2 h after both HIIT and MOD \((P < 0.01)\). Norepinephrine was also higher 1 h after MOD \((P < 0.01)\), whereas ACTH was lower than preexercise values 2 h after HIIT \((P < 0.01)\). Plasma insulin concentration did not change significantly following HIIT \((P = 0.14)\) or MOD \((P = 0.055)\) (Fig. 7G). However, during the postexercise recovery period, insulin was lower than preexercise values at 1 and 2 h after both HIIT and MOD \((P < 0.01)\). Plasma glucagon also did not change significantly during HIIT \((P = 0.12)\) or MOD \((P = 0.32)\) (Fig. 7H), whereas it was lower than preexercise values at 2 h after both HIIT and MOD \((P < 0.05)\).

**DISCUSSION**

In this study, we systematically compared the metabolic and hormonal responses to matched-work high-intensity interval and continuous moderate-intensity exercise. CHO oxidation, plasma glucose, and lactate were markedly higher after high-intensity interval exercise compared with continuous moderate-intensity exercise, whereas fat oxidation and total serum NEFA were not significantly different between the two exercise trials. Targeted metabolomic analysis of plasma samples revealed detailed insights into the exercise intensity-dependent changes in TCA cycle intermediates, specific NEFA, and amino acids. Although there was no significant difference in total serum NEFA between the two trials after exercise, we did notice some differences in the abundance of individual fatty acids. This finding highlights the importance of considering more than changes in total serum NEFA when evaluating the metabolic demands of exercise. The significant increase in
TCA cycle intermediates suggests that during exercise these metabolic compounds spill over from muscle into the circulation and/or are released systemically from other metabolic organs (e.g., the liver). The individual NEFA that were most responsive to exercise were not necessarily the most abundant NEFA in plasma. This finding raises some questions about the factors that determine the preference(s) for mobilization of NEFA during exercise. Furthermore, the changes in the percentage molar distribution of NEFA contrast with previous findings after exercise and challenge the previously held notion that changes in plasma NEFA composition reflect the composition of adipose tissue. These metabolic changes were accompanied by changes in counterregulatory hormones, some of which increased only after HIIT. Collectively, these findings highlight the value of targeted metabolomics to obtain more detailed and specific information on metabolic responses to exercise beyond basic measures of substrate oxidation, glucose, and free fatty acids.

TCA intermediates, including citric acid (1.8-fold), succinic acid (3.0-fold), aconitic acid (2.0-fold), and malonic acid (1.6-fold), all increased in plasma during high-intensity interval exercise in the present study. Of the TCA intermediates, only succinic acid increased in response to moderate-intensity exercise, and this response was considerably lower than the response following high-intensity interval exercise. Others have reported an increase in urinary citrate and α-ketoglutarate (48), urinary succinate (12), plasma/serum succinate, malate, fumarate, citrate-isocitrate, aconitic acid, and α-ketoglutarate (34, 42) after exercise. Lewis et al. (34) observed that succinate, malate, and fumarate (but not citrate-isocitrate, aconitic acid, or α-ketoglutarate) were higher in plasma from fast marathon runners compared with slow marathon runners. This finding also provides some indirect evidence of the intensity-dependent changes in these TCA intermediates after exercise that we observed. The biological role (if any) of TCA intermediates in urine and plasma after exercise is unclear because they are primarily active in skeletal muscle (14, 15) and the liver (24) during exercise. It is likely that the presence of TCA intermediates in urine and plasma after exercise likely reflects “spillover” from skeletal muscle or the liver.

The intensity-dependent increase in plasma TCA intermediates in the present study agrees with other reports that the concentration of TCA intermediates in skeletal muscle also increases with exercise intensity (15). The increase in the concentration of TCA intermediates in skeletal muscle, and possibly plasma too, during high-intensity exercise may result from epinephrine-induced accumulation of pyruvate (54). Gibala et al. (15) found that of all of the TCA intermediates in skeletal muscle, malate increased to the greatest extent (7.5 ×) during exercise and contributed the most to the expansion of the pool of TCA intermediates at the end of exercise. By contrast, succinic acid showed the greatest fold change in

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Fig. 5. Plasma amino acids following HIIT and MOD. Data are in arbitrary units and represent means ± SD. *P < 0.05 vs. pre-exercise. #P < 0.05 for HIIT vs. MOD.
plasma after exercise in the present study. One explanation for the rise in succinic acid is that carbon skeletons may enter the cycle at the level of 2-oxoglutarate through the alanine aminotransferase reaction, thereby causing accumulation of intermediates in the second span of the TCA cycle (i.e., malate, fumarate, succinate) (15). Typically, TCA intermediates accumulate in skeletal muscle early during exercise and then progressively decline thereafter (18). In the present study, the plasma concentrations of TCA intermediates may therefore have been higher during exercise compared with the end of exercise.

Most exercise studies have focused on changes in plasma/serum NEFA; comparatively little research has investigated exercise-induced changes in other lipid classes (e.g., phospholipids, mono-, di- or triacylglycerols, diacylglycerols, cholesteryl esters) (44). NEFA represent a relatively small proportion of fatty acids in tissues. Nevertheless, they are important in the context of exercise from both a physiological standpoint (i.e., alterations in the composition of fatty acids delivered to tissues) and a practical perspective (i.e., increasing the likelihood of detecting significant changes) (44). In addition to an increase in total serum NEFA, we detected increases in the plasma concentration of specific NEFA, including myristic acid (C14:0), decanoic acid (C10:0), dodecanoic acid (C12:0), hetadecenoic acid (C17:1), and palmitoleic acid (C16:1), after both exercise trials. Myristoleic acid (C14:1) and oleic acid (C18:1n-9) increased only after HIIT, whereas γ-linolenic acid (C18:3) increased only after continuous, moderate-intensity exercise.

Among the NEFA that increased after exercise in the present study, dodecanoic acid increased to the greatest extent (3.2-fold), palmitoleic acid and myristic acid increased moderately (2.5-fold), and oleic acid increased only slightly (1.3-fold). The percentage molar distribution of these NEFA also increased after exercise (Table 2). Interestingly, with the exception of oleic acid, these NEFA generally comprised <4% of the sum of all NEFA that we measured in plasma. Palmitoleic acid and myristic acid also make up only a small proportion of fatty acids in adipose tissue (23, 29). Changes in plasma fatty acids during exercise represent the balance between fatty acid release from adipose tissue and fatty acid uptake into skeletal muscle and the liver (41). There are several possible explanations as to why dodecanoic acid, palmitoleic acid, and myristic acid were preferentially mobilized during exercise despite their low relative abundance in plasma and adipose tissue. One possibility is that transport proteins for these particular fatty acids are more abundant in skeletal muscle and adipose tissue, and/or they are more sensitive to muscle contractions and hormones.
and palmitoleic acid were also still elevated 2 h after both heptadecenoic acid, dodecanoic acid, myristic acid, oleic acid, continuous, moderate-intensity exercise. Among the individual fatty acids, we found that total serum NEFA remained increased significantly only during high-intensity interval exercise. Furthermore, alanine was significantly higher after high-intensity exercise. The plasma concentrations of alanine, glutamate, and tyrosine increased significantly only during high-intensity interval exercise. The changes in these specific NEFA after exercise are generally consistent with other studies (6, 39). In addition to differences in the number of NEFA that we detected, the molar distribution of oleic acid and linoleic acid decreased after exercise. It is difficult to compare these studies with our results because we measured a greater number of plasma NEFA. Consequently, the ratio of unsaturated/saturated NEFA in plasma did not change after exercise, which contrasts with other research reporting an increase in this ratio. Mougios et al. (39) reported that the molar distribution of palmitic acid and stearic acid did not change, oleic acid increased slightly, and linoleic acid decreased slightly (Table 2). The changes in these specific NEFA after exercise are generally consistent with other studies (6, 39). In addition to differences in the number of NEFA that we detected, the variation between our findings and others (39) may be related to the intensity and duration of exercise and the sex and training status of the participants.

A strength of the present study is that we monitored metabolic changes not only immediately following completion of exercise but additionally during the early hours of recovery from exercise. We found that total serum NEFA remained elevated 2 h after both high-intensity interval exercise and continuous, moderate-intensity exercise. Among the individual fatty acids that we measured in plasma by metabolomics, heptadecenoic acid, dodecanoic acid, myristic acid, oleic acid, and palmitoleic acid were also still elevated 2 h after both exercise trials. Consistent with other studies, there were no significant differences in plasma fatty acid concentrations during recovery from the high-intensity exercise and the continuous moderate-intensity exercise (22, 41). In support of the present study in relation to postexercise lipid metabolism, several studies have found no difference in fat oxidation or respiratory exchange ratio after high-intensity exercise compared with moderate-intensity exercise (30, 31). By contrast, others have reported that fat oxidation was higher while respiratory exchange ratio was lower after high-intensity exercise compared with moderate-intensity exercise (36, 63). These discrepancies may reflect differences between these studies in exercise protocols and the training status of participants. It also remains unclear whether differences in intensity and substrate metabolism during exercise translate to differences in energy expenditure during recovery from exercise (31, 38).

In the present study, changes in the concentration of NEFA and other specific fatty acids (e.g., dodecanoic acid, myristic acid, palmitoleic acid) remained high for 2 h after exercise. Mulla et al. (41) reported similar changes in NEFA and glycerol output from adipose tissue during and after exercise. Together, these findings reflect a shift in substrate oxidation in favor of lipid metabolism (28, 30). This response may occur to continue supplying energy while facilitating restoration of glucose homeostasis and glycogen restoration (30).

This is the first study to compare the effects of exercise intensity on changes in a broad range of amino acids in plasma. The plasma concentrations of alanine, glutamate, and tyrosine increased significantly only during high-intensity interval exercise. Furthermore, alanine was significantly higher after high-intensity interval exercise compared with continuous moderate-intensity exercise. Other studies have reported an increase in alanine concentration in both venous plasma (3) and arterial plasma in response to exercise (40, 59). This rise in alanine within the circulation reflects its synthesis and release from skeletal muscle (58). Previous research indicates that alanine release from skeletal muscle is more sustained during exercise with low vs. normal muscle glycogen (59). We did not

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**Table 2. Percentage molar distribution of nonesterified fatty acids**

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Pre</th>
<th>Post</th>
<th>1 h</th>
<th>2 h</th>
<th>Pre</th>
<th>Post</th>
<th>1 h</th>
<th>2 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Octanoic acid (C8:0)</td>
<td>0.4 (0.3)</td>
<td>0.3 (0.3)</td>
<td>0.3 (0.1)</td>
<td>0.2 (0.1)</td>
<td>0.3 (0.1)</td>
<td>0.3 (0.1)</td>
<td>0.2 (0.1)*</td>
<td>0.2 (0.1)*</td>
</tr>
<tr>
<td>Decanoic acid (C10:0)</td>
<td>0.3 (0.2)</td>
<td>0.5 (0.3)</td>
<td>0.5 (0.3)</td>
<td>0.4 (0.3)</td>
<td>0.4 (0.2)</td>
<td>0.6 (0.2)</td>
<td>0.4 (0.2)</td>
<td>0.5 (0.3)</td>
</tr>
<tr>
<td>Palmitoleic acid (C16:1)</td>
<td>0.2 (0.2)</td>
<td>1.5 (0.6)*</td>
<td>1.0 (0.8)</td>
<td>1.3 (0.9)*</td>
<td>0.7 (0.4)</td>
<td>1.8 (0.5)*</td>
<td>1.1 (0.6)*</td>
<td>1.3 (0.8)*</td>
</tr>
<tr>
<td>Myristic acid (C14:0)</td>
<td>1.8 (0.5)</td>
<td>3.4 (0.9)*</td>
<td>2.7 (1.0)*</td>
<td>3.4 (1.6)*</td>
<td>2.0 (0.7)</td>
<td>4.4 (1.2)*</td>
<td>3.1 (0.9)*</td>
<td>3.3 (1.0)*</td>
</tr>
<tr>
<td>Palmitic acid (C16:0)</td>
<td>30 (0.8)</td>
<td>29 (0.8)</td>
<td>30 (0.8)</td>
<td>30 (0.8)</td>
<td>32 (5.8)</td>
<td>29 (1.3)</td>
<td>30 (0.7)</td>
<td>30 (1.0)</td>
</tr>
<tr>
<td>Margaric acid (C17:0)</td>
<td>1.1 (0.2)</td>
<td>1.3 (0.3)</td>
<td>1.2 (0.2)</td>
<td>1.2 (0.3)</td>
<td>1.0 (1.0)</td>
<td>1.2 (0.3)</td>
<td>1.2 (0.2)</td>
<td>1.3 (0.2)</td>
</tr>
<tr>
<td>Stearic acid (C18:0)</td>
<td>24 (1.2)</td>
<td>23 (1.2)</td>
<td>23 (1.2)</td>
<td>23 (1.2)</td>
<td>22 (3.5)</td>
<td>21 (2.2)</td>
<td>21 (1.6)</td>
<td>22 (1.0)</td>
</tr>
<tr>
<td>Arachidic acid (C20:0)</td>
<td>0.6 (0.4)</td>
<td>0.6 (0.4)</td>
<td>0.6 (0.4)</td>
<td>0.6 (0.4)</td>
<td>0.6 (0.4)</td>
<td>0.6 (0.4)</td>
<td>0.6 (0.4)</td>
<td>0.6 (0.4)</td>
</tr>
<tr>
<td>Myristoleic acid (C14:1)</td>
<td>0.02 (0.004)</td>
<td>0.03 (0.005)</td>
<td>0.02 (0.005)</td>
<td>0.02 (0.003)</td>
<td>0.06 (0.127)</td>
<td>0.02 (0.005)</td>
<td>0.02 (0.003)</td>
<td>0.03 (0.004)</td>
</tr>
<tr>
<td>Palmitoleic acid (C16:1)</td>
<td>0.3 (0.1)</td>
<td>0.6 (0.2)*</td>
<td>0.5 (0.2)*</td>
<td>0.7 (0.3)*</td>
<td>0.4 (0.1)</td>
<td>0.9 (0.4)*</td>
<td>0.6 (0.2)*</td>
<td>0.7 (0.2)*</td>
</tr>
<tr>
<td>Vaccenic acid (C18:1n-7)</td>
<td>16 (0.9)</td>
<td>15 (1.1)*</td>
<td>16 (1.3)</td>
<td>15 (0.9)*</td>
<td>16 (1.6)</td>
<td>15 (1.8)</td>
<td>15 (1.1)</td>
<td>15 (0.8)</td>
</tr>
<tr>
<td>Palmitoleic acid (C16:1)</td>
<td>7.5 (1.0)</td>
<td>8.1 (1.0)</td>
<td>7.9 (0.9)*</td>
<td>8.7 (1.1)</td>
<td>8.8 (1.0)</td>
<td>8.8 (0.7)</td>
<td>8.2 (0.7)</td>
<td>8.3 (0.7)</td>
</tr>
<tr>
<td>Linoleic acid (C18:2)</td>
<td>16 (0.9)</td>
<td>15 (1.1)*</td>
<td>16 (1.3)</td>
<td>15 (0.9)*</td>
<td>16 (1.6)</td>
<td>15 (1.8)</td>
<td>15 (1.1)</td>
<td>15 (0.8)</td>
</tr>
<tr>
<td>γ-Linolenic acid (C18:3)</td>
<td>0.7 (0.3)</td>
<td>0.8 (0.2)</td>
<td>0.7 (0.2)</td>
<td>0.7 (0.3)</td>
<td>0.7 (0.5)</td>
<td>0.9 (0.3)</td>
<td>0.7 (0.2)</td>
<td>0.7 (0.2)</td>
</tr>
<tr>
<td>Arachidonic acid (C20:4)</td>
<td>5.4 (0.8)</td>
<td>4.8 (0.8)</td>
<td>5.1 (0.9)</td>
<td>4.7 (0.6)</td>
<td>4.8 (1.3)</td>
<td>4.6 (0.7)</td>
<td>4.9 (0.7)</td>
<td>4.9 (0.7)</td>
</tr>
<tr>
<td>Eicosapentaenoic acid (C20:5)</td>
<td>0.9 (0.3)</td>
<td>0.8 (0.3)</td>
<td>0.8 (0.3)</td>
<td>0.8 (0.2)</td>
<td>1.0 (0.3)</td>
<td>0.9 (0.4)</td>
<td>0.9 (0.4)</td>
<td>1.0 (0.3)</td>
</tr>
<tr>
<td>Docosahexaenoic acid (C22:6)</td>
<td>1.9 (0.5)</td>
<td>1.9 (0.6)</td>
<td>1.8 (0.4)</td>
<td>1.8 (0.3)</td>
<td>1.8 (0.5)</td>
<td>1.7 (0.6)</td>
<td>1.8 (0.5)</td>
<td>1.9 (0.4)</td>
</tr>
<tr>
<td>Adrenic acid (C22:4)</td>
<td>0.15 (0.07)</td>
<td>0.14 (0.04)</td>
<td>0.14 (0.03)</td>
<td>0.13 (0.02)</td>
<td>0.24 (0.35)</td>
<td>0.13 (0.03)</td>
<td>0.13 (0.02)</td>
<td>0.14 (0.04)</td>
</tr>
<tr>
<td>Unsaturated-to-saturated ratio</td>
<td>0.73 (0.04)</td>
<td>0.70 (0.03)</td>
<td>0.72 (0.04)</td>
<td>0.71 (0.04)</td>
<td>0.72 (0.04)</td>
<td>0.69 (0.03)</td>
<td>0.71 (0.04)</td>
<td>0.70 (0.04)</td>
</tr>
</tbody>
</table>

*Significant difference vs. preexercise, P < 0.05.
measure muscle glycogen in the present study, but the greater reliance on CHO metabolism could possibly account for the higher plasma concentration of alanine after high-intensity interval exercise. In contrast with our findings, other studies have reported a decrease (40) or no change (59) in the concentration of glutamate in arterial plasma. In skeletal muscle, glutamate consumption increases markedly during the first few minutes of exercise, particularly when muscle glycogen is low (59). The consumption of glutamate in muscle may shift the alanine aminotransferase reaction in favor of the formation of alanine and TCA intermediates such as α-oxoglutarate (58). We can only speculate, but the increase in venous plasma glutamate concentration that we observed may have served to generate more TCA intermediates during high-intensity interval exercise. We detected increases in the plasma concentrations of 4-methyl-2-oxopentanoic acid and 3-methyl-2-oxopentanoic acid after high-intensity intermittent exercise. These carboxylic acids may represent breakdown products of leucine (46).

The other amino acids that we measured in plasma (i.e., threonine, phenylalanine, glycine, cysteine, proline, and ornithine) did not change significantly during either exercise trial.

Fig. 7. Plasma hormones following HIIT and MOD. Data represent means ± SD for glucagon; geometric mean ± 95% confidence interval for ACTH, insulin, and growth hormone; and median ± interquartile range for norepinephrine, epinephrine, cortisol, and interleukin (IL)-6. *P < 0.05 vs. preexercise. #P < 0.05 for HIIT vs. MOD. NB, free cortisol represents the ratio between total cortisol and cortisol-binding globulin.
Some of these amino acids, such as threonine, phenylalanine, and glycine, are not metabolized in skeletal muscle (58), which could explain why they did not change after exercise in this study. It is possible that phenylalanine is converted to tyrosine, which might partially account for the rise in plasma tyrosine concentration that occurred during high-intensity interval exercise.

During recovery from exercise, branched-chain amino acids, methionine, alanine, and proline were all significantly lower compared with preexercise, particularly after continuous moderate-intensity exercise. This sustained decline in these amino acids during recovery from exercise reflects their role in gluconeogenesis. Wahren et al. (61) provided early evidence that amino acids (particularly alanine), pyruvate and lactate assist in restoring hepatic glucose metabolism after exercise. More recent work by Mourtzakis et al. (40) revealed that efflux of branched-chain amino acid, glutamine, and alanine from skeletal muscle stimulates a significant rise in arterial blood glucose concentration during recovery from exercise. Despite a difference in plasma glucose concentration after exercise, the plasma concentrations of amino acids during recovery were not significantly between the two exercise trials in the present study. Conceivably, amino acids may play a more significant role in supporting gluconeogenesis during recovery from exercise that depletes rather than raises blood glucose concentration (40).

We detected an increase in several other metabolic species in plasma, including 2-hydroxybutyric acid, itaconic acid, and citraconic acid, most notably after high-intensity intermittent exercise. These metabolites do not fall into any of the classical metabolic clusters, and relatively little is known about their biological functions, at least in humans. However, 2-hydroxybutyric acid is elevated in urine from patients with lactic acidosis and ketoacidosis (32), which suggests that it plays some sort of role under conditions of metabolic stress. Iaconic acid may be derived from aconitic acid (which is a TCA cycle intermediate), whereas citraconic acid may be a by-product of glutamine metabolism (47).

We attempted to link intensity-dependent changes in metabolism with specific hormonal responses. ACTH, cortisol, and growth hormone were significantly higher after high-intensity intermittent exercise compared with continuous moderate-intensity exercise. These metabolites do not fall into any of the classical metabolic clusters, and relatively little is known about their biological functions, at least in humans. However, 2-hydroxybutyric acid is elevated in urine from patients with lactic acidosis and ketoacidosis (32), which suggests that it plays some sort of role under conditions of metabolic stress. Iaconic acid may be derived from aconitic acid (which is a TCA cycle intermediate), whereas citraconic acid may be a by-product of glutamine metabolism (47).

Catecholamines and IL-6 may also have induced a rise in plasma glucose during exercise (27, 35). Exercise suppressed insulin release, whereas plasma glucagon concentration did not change significantly after exercise. It is possible that ~1 h exercise was not long enough, and/or the catecholamine response to exercise was not sufficient to induce the release of glucagon (13). Either way, it would appear that glucagon played a minor role (if any) in regulating glucose metabolism during exercise in the present study.

Cortisol, growth hormone, and catecholamines stimulate lipolysis during exercise (1, 11, 26). However, because the plasma concentrations of NEFA were not significantly different after high-intensity intermittent exercise vs. continuous moderate-intensity exercise, it is difficult to assign a specific role for these hormones in regulating fat metabolism during exercise. At rest, IL-6 induces lipolysis in skeletal muscle (but not adipose tissue) (66), yet its role in regulating lipid metabolism during exercise remains unresolved (62). Cortisol mediates protein metabolism during exercise (11) and could possibly account for the higher plasma alanine concentration after high-intensity intermittent exercise in the present study.

There were several limitations to the present study. First, we could not determine between pre- and postexercise data for both trials, the results of the two exercise trials are not directly comparable. In summary, the present study provides detailed insights into the metabolic and hormonal responses to high-intensity interval exercise, which is rapidly gaining popularity as a time-effective form of training. Our findings highlight the benefits of a targeted metabolomic approach to characterize changes in metabolism through the TCA cycle and lipid and protein pathways. The athletes in the present study consumed breakfast before exercise; the metabolic response to increase may be different under fasting conditions. Future research could use a targeted metabolomic approach to investigate differences in metabolism relative to individual thresholds for maximal fat oxidation. We only measured a small subset of the array of possible ligands. Future studies employing targeted metabolomics analysis could investigate changes in other ligands, including triglycerides, phospholipids, and cholesterol esters. Further research is also needed to examine metabolomics adaptations to exercise training.


