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Acute high-intensity exercise and skeletal muscle mitochondrial respiratory function: role of metabolic perturbation

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Abstract

Recently it was documented that fatiguing, high-intensity exercise resulted in a significant attenuation in maximal skeletal muscle mitochondrial respiratory capacity, potentially due to the intramuscular metabolic perturbation elicited by such intense exercise. With the utilization of intrathecal fentanyl to attenuate afferent feedback from group III/IV muscle afferents, permitting increased muscle activation and greater intramuscular metabolic disturbance, this study aimed to better elucidate the role of metabolic perturbation on mitochondrial respiratory function. Eight young, healthy males performed high-intensity cycle exercise in control (CTRL) and fentanyl-treated (FENT) conditions. Liquid chromatography-mass spectrometry and high-resolution respirometry were used to assess metabolites and mitochondrial respiratory function, respectively, pre- and postexercise in muscle biopsies from the vastus lateralis. Compared with CTRL, FENT yielded a significantly greater exercise-induced metabolic perturbation (PCr:–67% vs. –82%, Pi: 353% vs. 534%, pH: –0.22 vs. –0.31, lactate: 820% vs. 1,160%). Somewhat surprisingly, despite this greater metabolic perturbation in FENT compared with CTRL, with the only exception of respiratory control ratio (RCR) (-3% and –36%) for which the impact of FENT was significantly greater, the degree of

attenuated mitochondrial respiratory capacity postexercise was not different between CTRL and FENT, respectively, as assessed by maximal respiratory flux through complex I (-15% and –33%), complex II (-36% and –23%), complex I þ II (-31% and –20%), and state $3_{CI\ þ\ CII}$ control ratio (-24% and –39%). Although a basement effect cannot be ruled out, this failure of an augmented metabolic perturbation to extensively further attenuate mitochondrial function questions the direct role of high-intensity exercise-induced metabolite accumulation in this post- exercise response.

mitochondrial function; muscle afferents; muscle metabolites; oxidative phosphorylation

INTRODUCTION

Recently, our group documented the negative effect of high-intensity exercise on skeletal muscle mitochondrial respiratory function (1). Skeletal muscle respiratory capacity, assessed as maximal ADP-stimulated respiration, decreased >35% following exhaustive cycling exercise, assessed with high-resolution respirometry utilizing permeabilized muscle fibers, sampled from the vastus lateralis (1). Although some- what speculative, several putative mechanisms responsible for the high-intensity exercise-induced attenuation in oxidative phosphorylation capacity were proposed. Specifically, potential candidates included: *1*) diminished substrate avail- ability because of the impaired activity of key enzymes of the tricarboxylic acid (TCA) cycle; *2*) free radical accumulation, in particular hydrogen peroxide; *3*) muscle contraction generated heat, and, finally; *4*) the accumulation of metabolites, such as inorganic phosphate (Pi), [lactate], and protons (1). Although each of these candidates had grounds for con- sideration, this prior study did not provide a definitive link between any of these putative mechanisms and the exercise-induced impairment in mitochondrial respiratory function.

Of all the responses to high-intensity exercise, metabolic perturbation is a prime suspect for negatively impacting mitochondrial respiratory function as its severity parallels relative exercise intensity across all levels of fitness (2–7).

Indeed, as exercise intensity increases, all working skeletal muscle is confronted with large alterations in the chemical environment, including elevated concentrations of muscle metabolites in direct proportion to exercise intensity (8, 9). Concomitantly, high metabolite concentrations, reflecting high-intensity exercise, are documented to impair mitochondrial respiration in isolated conditions (10–12). For example, skeletal muscle mitochondrial respiratory function was attenuated in vitro after exposing isolated mitochondria to elevated levels of acidity (pH = 6.38) (10) or permeabilized fibers to elevated inorganic phosphate (20 mM) (11) quantified by a reduction in respiratory control ratio (RCR) and creatine-stimulated respiration, respectively. Similar effects of metabolic perturbation are documented under isolated conditions in vivo, as evidenced by >50% attenuated respiratory capacity following hypercapnia-induced acidosis in the isolated muscles of the cat hindlimb, measured by both phosphorus magnetic resonance spectroscopy and utilizing the direct Fick approach (12). Although the negative impact of metabolite accumulation on metabolism is apparent in isolated conditions, the impact of metabolite accumulation on mitochondrial respiratory function following whole body, high-intensity exercise in humans is worthy of greater study.

Skeletal muscle metabolite concentrations are tightly regulated during exercise and therefore reach similar levels at exhaustion in the severe intensity domain (13–15). This is, at least in part, because of restrained muscle activation mediated by group III/IV muscle afferents (15). Recently, our group has utilized lumbar intrathecal fentanyl administration during whole body exercise to impair the projection from group III/IV muscle afferents to the central nervous system and increase lower limb muscle activation (16–18). More specifically, the increased muscle activation, facilitated by the partial blockade of group III/IV muscle afferents with fentanyl, resulted in an increased accumulation of muscle metabolites during exhaustive cycling exercise (18). Utilizing this approach, it is possible to evaluate the direct impact of augmenting the intramuscular metabolic perturbation during high-intensity exercise on skeletal muscle mitochondrial respiratory function.

Accordingly, the aim of this study was to elucidate the role of intramuscular perturbation on the previously established impact of acute high-intensity exercise on skeletal muscle mitochondrial respiratory function by partially blocking group III/IV muscle afferents using intrathecal fentanyl administration. We hypothesized that the greater metabolic perturbation, facilitated by fentanyl administration, during high-intensity exercise would result in a more pronounced impairment in mitochondrial respiratory function when compared with high-intensity exercise in control conditions. Furthermore, we hypothesized that the exercise-induced change in metabolic perturbation would be related to the change in mitochondrial respiratory function.

METHODS

Participants

Eight healthy, recreationally active males, with no known metabolic, neurological, or cardiovascular disease, volunteered to participate in this study. Written informed consent was obtained from each participant before the beginning of the study. All experimental procedures were approved by the University of Utah and the Salt Lake City Veterans Affairs Medical Center Institutional Review Boards and con- formed to the Declaration of Helsinki. The intramuscular metabolite data (18) and mitochondrial respiratory flux pre- and postexercise without fentanyl treatment (1) for the cur- rent subjects have been published previously, in a different form, but are presented again here as they are integral to the current investigation.

Experimental Protocols

Exercise.

The exercise trials were performed as previously described by Blain et al. (18). Briefly, subjects were thoroughly familiarized with the procedures used during preliminary visits to the laboratory. All participants performed a practice 5-km cycling time trial and a maximal incremental exercise test [20 W þ 25 W/min; (19)] on a computer-controlled electro- magnetically braked cycle

ergometer (Velotron, Elite Model, Racer Mate, Seattle, WA) for the determination of peak power output (W_{peak}) and maximal oxygen consumption ($\dot{V}O_{2max}$). On separate days, in random order, all participants performed two 5-km cycling time trials (20): a control time trial with no treatment (CTRL), and an experimental time trial with intrathecal fentanyl (1 mL volume, 0.025 mg/mL) applied at vertebral interspace L3-L4 (FENT). Fentanyl is an opioid analgesic, which when given at L3-L4, binds to spinal opioid receptors and attenuates ascending group III/IV fibers that are responsible for cortical projection of pain, metabolic accumulation, and mechanical contraction. Because of the specificity and lipid-soluble properties of the injection, fentanyl is quickly absorbed near the site of injection and, thus, localized to L3–L4 of the spinal column. Localization has been evidenced by no cephalad migration, localized hypoalgesia, and absence of fentanyl in venous blood measured 10 and 50 min postinjection at this dosage (16). Thus, at this dose, the participants' mental state is at minimal risk for any alteration outside of lessened perception of pain or tiredness from muscle below the L3-L4 region and off-target effects are avoided. The subjects remained seated throughout exercise. To avoid initial peak power outputs, subjects were instructed to pick up their pace slowly, and the recording period began after the starting power output and pedal cadence, adopted from the practice time trial, were reached (within 10–15 s). Once recording commenced, subjects were free to alter power output by changing the gear ratio and/or pedaling frequency. Each exercise session was separated by at least 72 h, completed at the same time of day, with ambient temperature and relative humidity maintained constant between the two conditions. Subjects were instructed to refrain from caffeine for 12 h and exercise for 48 h before each exercise trial.

Pulmonary gas exchange measurements.

At rest and throughout exercise, pulmonary gas exchange and ventilation were measured continuously using an open circuit calorimetry system (Parvo Medics, True Max 2400, Salt Lake City, UT).

Arm cranking test.

Any migration of fentanyl from its intrathecal application at the lumbar level to beyond the cervical level would complicate interpretation of the findings. Therefore, as the binding of fentanyl to medullary opioid receptors would attenuate the ventilatory response to upper body exercise, the ventilatory response to arm cranking (15 and 30 W for 3 min each; Monark-Crescent AB, Varberg, Sweden) was assessed before and 10 min after fentanyl injection to evaluate whether a cephalad drug migration to the brain occurred(21). To ensure similar pretime trial procedures, this arm cranking test was also implemented during CTRL.

Muscle biopsies.

A muscle sample of the vastus lateralis (VL) was obtained, under sterile conditions, before (pre) and immediately after (post) the 5-km time trial in both the CTRL and FENT conditions by a percutaneous needle biopsy ~15 cm proximal to the knee at a depth of ~3.5 cm (22). Of note, the pre-FENT biopsy was taken before the administration of fentanyl to avoid losing the effect of fentanyl before the completion of exercise. Fentanyl's effect begins wearing off within 1 h after administration, and given the time required after the pre- biopsy for setup, arm crank testing, and exercise, administration before the pre-FENT biopsy was not feasible. However, given the localization of fentanyl as described earlier, any independent effect of fentanyl on mitochondrial respiration is unlikely. The right leg was used for pre-exercise sampling, whereas the left leg was used for postexercise sampling, and this order was maintained across visits. During the second visit, biopsies were taken along the same longitudinal axis of the VL, but 2–3 cm below the biopsy site from the first experimental session. Immediately after the exercise, an occlusion cuff was rapidly inflated on the upper part of the thigh and maintained at suprasystolic pressure (250 mmHg) to clamp the metabolic milieu until muscle sampling was complete (<30 s postexercise). Previous work has determined that 250 mmHg of cuff pressure is adequate for complete occlusion (23) and that postexercise occlusion prevents any increase or decrease in

metabolites, effectively clamping the metabolic perturbation (24–27). Furthermore, the relatively short time of ischemia compared with that documented to induce mitochondrial dysfunction (28), along with the relatively large O_2 storage capacity within the muscle (24, 29), likely prevented the cuff application from causing an anoxic state and, thus, would not hinder mitochondrial respiratory function. Immediately after the muscle sample (~100 mg) was taken from the leg, part of the sample (~20 mg) was immersed in ice-cold biopsy preservation fluid [BIOPS, in mM: 2.77 CaK_2EGTA, 7.23 K_2EGTA, 20.0 imidazole, 50.0 K^{þ}-MES, 20.0 taurine, 0.5 dithiothreitol, 6.56 $MgCl_2$, 5.77 ATP, 15.0 phosphocreatine (PCr), pH 7.1 at 4°C] for respiratory analyses (30), whereas the remaining tissue was flash frozen in liquid nitrogen and stored at –80°C for later analyses.

Mitochondrial respiration.

Muscle samples were prepared and permeabilized for mitochondrial respiration analysis as described by Pesta and Gnaiger (30). Briefly, BIOPS-immersed fibers were carefully separated with fine-tip forceps and subsequently bathed in a BIOPS-based saponin solution (50 µg saponin/mL BIOPS) for 30 min. After saponin treatment, muscle fibers were rinsed twice in ice-cold mitochondrial respiration fluid (MiR05, in mM: 110 sucrose, 0.5 EGTA, 3 $MgCl_2$, 60 K^{þ}-lactobionate, 20 taurine, 10 KH_2PO_4, 20 HEPES, in distilled water with 1 g/L BSA, pH 7.1 at 37°C) for 10 min each rinse. After being rinsed for a total of 20 min, fibers were blotted with a paper towel to measure the weight of each sample (2–4 mg). Muscle fibers were then placed in the temperature-controlled respiration chamber (Oxytherm, Hansatech Instruments, Norfolk, UK) in 2 mL MiR05 solution and warmed to 37°C. The respiration chamber was calibrated daily, and MiR05 was air saturated with O_2 concentrations of ~190 to ~175 µM O_2 before start of the experiment. After 10 min of equilibration, mitochondrial respiratory flux was quantified by: *1*) non-phosphorylating (no ADP added) basal leak respiration driven by complex I (CI, state 2_{CI}), *2*) ADP-stimulated peak CI-driven respiration (state 3_{CI}), peak complex II (CII)-driven respiration (state 3_{CII}), and peak respiration from

convergent electron flow through both CI þ CII (state $3_{CI\ þ\ CII}$), and *3*) uncoupled (direct complex IV activation) respiration (UC_{CIV}) using the protocol described in Table 1. Pilot studies indicated that experimental substrate and inhibitor concentrations were at saturating levels (31).

Only samples that exhibited evidence of mitochondrial membrane integrity (<10% increase in respiration with addition of cytochrome *c*) were included in this study. Data were analyzed from the average respiration over the final minute of steady-state respiration for each step, averaged across duplicate runs, and presented as O_2 flux per mg of tissue (wet weight). Given the within-subject comparison design, the rate of O_2 consumption was expressed relative to muscle sample mass, as mitochondrial content is unlikely to change within such a short time frame (32). The state 2_{CI} control ratio [i.e., the ratio between state 2_{CI} leak and UC_{CIV} respiration, a similar, but lower value compared with Pesta and Gnaiger's LEAK control ratio due to UC_{CIV} exceeding uncoupled electron transport system (ETS) flux] was calculated as an index of dyscoupling relative to maximal complex IV capacity due to electron leak across the membrane, slip of protons in the respiratory chain, or reduced ETS capacity (30). The state $3_{CI\ þ\ CII}$ control ratio (i.e., the ratio between state $3_{CI\ þ\ CII}$ and UC_{CIV} respiration, again similar but lower compared with Pesta and Gnaiger's ROUTINE control ratio) was calculated as an index of the excess capacity of cytochrome *c* oxidase, representative of phosphorylative or ETS flux limitations to respiration (30, 33–35). Finally, the respiratory control ratio (RCR), an indicator of mitochondrial coupling efficiency and quality, was quantified by state 3_{CI}/state 2_{CI} (30, 36). State 3_{CI} and state 2_{CI} were used as the most accurate calculation of RCR as, in this protocol (Table 1), state $2_{CI\ þ\ CII}$ and state 2_{CII} were not assessed without the addition of ADP, and state 4 was not quantified due to use of rotenone to inhibit complex I. Of note, calculating RCR using state 3_{CII} or state $3_{CI\ þ\ CII}$ would not have been valid if quantified using the lower, CI-only state 2 (2, 36).

Citrate synthase activity.

The same muscle samples used for respiration were subsequently tested for citrate synthase (CS) activity, as described previously (37, 38), to estimate mitochondrial content. Samples were prepared by, first, homogenizing in homogenization buffer [in mM: 250 sucrose, 40 KCl, 2 EGTA, and 20 Tris·HCl (Qiagen, Hilden, Germany)], and then the CS activity assay was performed by spectrophotometry at 412 nm absorbance (Biotek Instruments, Winooski, VT).

Table 1. *Mitochondrial respiration protocol*

Step	Chemical Name (Concentration)	Major Site of Action	Respiration State
1	Malate (2 mM), glutamate (10 mM)	+ Complex I (CI)	State 2_{CI}
2	ADP (5 mM)	+ Complex V (CV)	State 3_{CI}
3	Succinate (10 mM)	+ Complex II (CII)	State $3_{CI + CII}$
4	Cytochrome c (10 µM)	Test of mitochondrial membrane integrity	
5	Rotenone (0.5 µM)	-CI	State 3_{CII}
6	Antimycin A (2.5 µM), oligomycin (2 µM), TMPD (2 mM), ascorbate (0.5 mM)	-CIII, -CV, þ CIV	$Uncoupled_{CIV}$

Description of the protocol used to assess mitochondrial respiration, the site of action of each chemical introduced to the preparation (þ substrate; – inhibitor), and the respiration state associated with each step. Note that steady-state rates were achieved for each step, which took approximately 3 min, before proceeding to the next step. TMPD, *N*,*N*,*N*^{0},*N*^{0}-tetramethylphenylenediamine.

Muscle Metabolite Methodologies

Muscle sample extraction method.

Muscle samples were kept at –80°C until the day of extraction. On the day of extraction, the sample was transferred to a tube prefilled will 1.4-mm diameter ceramic beads, weighed, and returned to the freezer. Subsequently, the sample tube was vigorously beaten in an Omni Bead Ruptor Homogenizer at 6.45 mHz for 15 s (Omni International, Kennesaw, GA). After homogenization, 400 mL extraction solution (2:1 acetonitrile: water with 0.1% formic acid) containing internal standards of [^{13}C]ATP and d4-succinate (5 mg/ mL) was added. The tube was beaten again at 6.45 mHz for 15 s and incubated at 4°C for 15 min. The sample was then centrifuged at 20,000 *g* for 10 min at 4°C to pellet the tissue debris. The resulting supernatant was collected, and 3 mL was injected into the liquid chromatography

mass spectrometer (LC-MS) (1290 UPLC, Agilant, Santa Clara, CA) for analysis.

LC-MS analysis.

A 1290 UPLC (Agilent) fitted with a 100 x 2.1 mm Sequant ZIC-pHILIC column (Merck, Dramstadt, Germany) was employed for chromatographic separation of metabolites. Throughout the protocol, the liquid chromatograph column compartment was held at 10°C and the flow rate maintained at 0.2 mL/min. HPLC mobile phases used were *A* = 95% acetonitrile, 5% H_2O with 0.1% ammonium hydroxide; *B* = 10 mM ammonium formate in H_2O, pH 9.2. HPLC gradient used (*t* = 0 min) 100% *A*, 0% *B*; (*t* = 0.5 min) 100% *A*, 0% *B*; (*t* = 17.0 min) 62% *A*, 38% *B*; (*t* = 18.0 min) 42% *A*, 58% *B*; (*t* = 18.5 min) 42% *A*, 58% *B*; (*t* = 19.0 min) 100% *A*, 0% *B*; (*t* = 26.0 min) 100% *A*, 0% *B*. An Agilent 6550 iFunnel Q-ToF system was used for detection of eluted metabolites. The MS detection parameters were as follows: the instrument was operated in the negative mode; gas temperature 290°C; drying gas flow 11 L/min; nebulizer pressure 60 psig; sheath gas temperature 290°C; sheath gas flow 12 L/min; capillary volt- age 2,000 V; nozzle voltage 0 V; fragmentor 175 V; skimmer 65 V; OCT1 RF Vpp 750 V.

LC-MS data analysis.

The *m/z* values were calculated based on the corresponding metabolite formula as $[M-H]^-$ anion species. The *m/z* window used for spectral extraction was 35 ppm. Extracted spectra were smoothed, integrated, and area under the curve was used for analysis. Data were processed using the MassHunter software package (Agilent). All data were normalized to their respective internal standard and tissue mass. Because of a phenomenon known as ion suppression, absolute values of metabolite concentrations were not attainable, and thus are expressed as a relative change compared with resting baseline (18). The pH measurement was made on the muscle homogenate with a microelectrode (MI-415, Microelectrodes Inc., Bedford, NH) connected to a pH meter (SA 520, Orion Research Inc., Cambridge, MA).

Statistical Analyses

Comparisons of group means were performed by paired Student's *t* test for $\dot{V}O_2$, power, metabolite changes, and per- cent change in the assessments of mitochondrial respiration. For $\dot{V}O_2$, comparisons between CTRL and FENT were made after first averaging the data across 1–2.5 km or 2.6–5 km within each condition, representing the first and second portions of exercise, respectively. The first portion of exercise excluded 0–1 km for $\dot{V}O_2$ to avoid the variability from the $\dot{V}O_2$ on-kinetics and was compared only during the plateau.

Similarly, power was initially averaged within each condition across 0–2.5 km or 2.6–5 km, representing the first and second halves of exercise for subsequent comparison between CTRL and FENT. In accordance with our hypothesis, percent changes in metabolites and mitochondrial respiration were analyzed by one-tailed paired Student's *t* test with the a priori expectation of greater accumulation and attenuation, respectively. Two-way repeated-measures ANOVA (treatment x time) was used for respirometric analyses. When a significant difference was identified, post hoc analysis was performed using Tukey's honestly significant difference test. Correlations between variables were assessed with a Pearson's product-moment correlation. Of note, to abide by the assumption of independence of cases for a Pearson's correlation, the average of the CTRL and FENT values for each participant was used to avoid duplicate measures per individual. All statistical analyses were performed using SigmaPlot software (San Jose, CA). For all analyses, P ::; 0.05 was considered significantly different. All data are presented as means ± SE.

RESULTS

Participants

All eight of the participants completed the study without incident. They were 26 ± 2 yr of age, had a body mass of 83 ± 15 kg, and a height of 181 ± 5 cm. Their $\dot{V}O_{2max}$, assessed on a cycle ergometer, was 44 ± 7 mL/kg/min and they

produced a W_{peak} of 296 ± 37 W. There was no evidence of the migration of fentanyl beyond the cervical level in any participant as pulmonary ventilation during arm cranking at 15 W and 30 W was unchanged between CTRL and FENT.

Time Trial Performance

Pulmonary $\dot{V}O_2$ and power output throughout the 5-km time trial are illustrated in Fig. 1. Mean $\dot{V}O_2$ was significantly elevated in the FENT condition from 1 to 2.5 km (CTRL: 3.01 ± 0.10; FENT: 3.22 ± 0.13 L/min), which corresponded with significantly increased power output during the first half of the exercise in this condition (CTRL: 221 ± 9; FENT: 243 ± 13 W). There was no difference between treatment conditions in mean $\dot{V}O_2$ (CTRL: 3.06 ± 0.11; FENT: 3.14 ± 0.13 L/min) and mean power output (CTRL: 220 ± 9; FENT: 220 ± 12 W) over the duration of the 5-km time trial. Time to completion of the 5-km time trial was not different between conditions (CTRL: 8.8 ± 0.4; FENT: 8.8 ± 0.5 min).

Muscle Metabolites

Intrathecal fentanyl administration resulted in an increased muscle metabolic perturbation compared with CTRL during the 5-km time trial (Fig. 2). Specifically, relative to resting baseline, significantly more PCr was hydrolyzed in FENT compared with CTRL, which corresponded with significantly increased Pi accumulation in FENT compared with CTRL. Furthermore, intramuscular lactate concentration in exercised muscle, relative to resting baseline, was significantly increased in FENT compared with CTRL, which corresponded with a significantly greater change in intramuscular pH from baseline to end exercise. A complete summary of the LC-MS metabolite results are presented in Table 2. Of note, the reduced “n” for metabolites were the result of technical difficulties in the LC-MS analyses and, thus, were not quantified in two subjects.

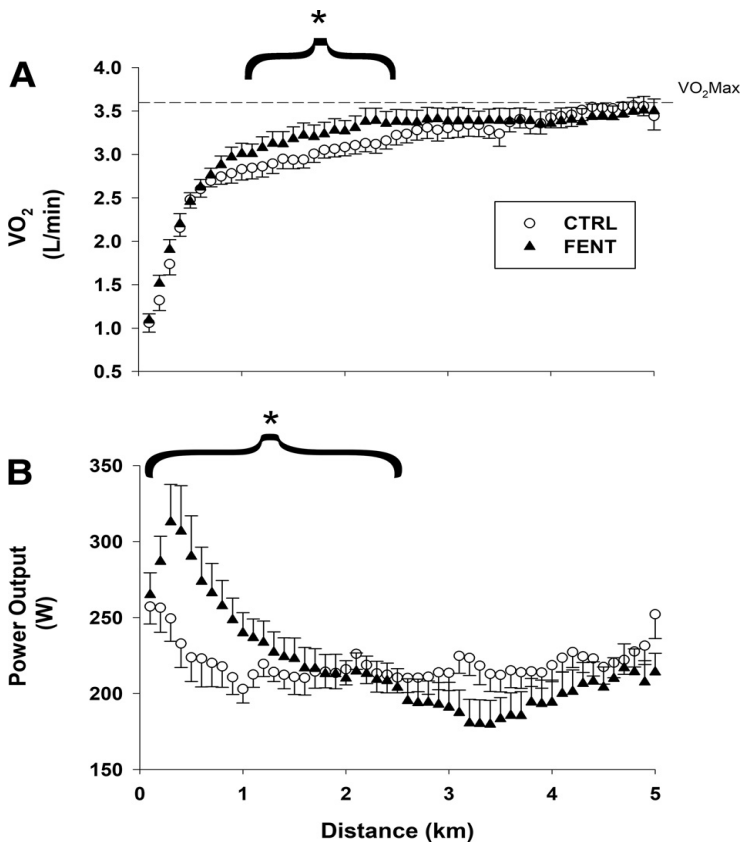

Figure 1. Pulmonary oxygen consumption ($V\dot{}O_2$, *A*) and power output (*B*) during 5-km cycling time trials in control (CTRL, white circles) and fentanyl- treated (FENT, black triangles) conditions. Although mean $V\dot{}O_2$ and power output were not different over the course of the exercise, FENT resulted in significant elevation in both $V\dot{}O_2$ and power output during the first half of exercise. Values are means ± SE. $^*P \leq 0.05$, paired Student's *t* test, subjects $n = 8$. *B* was reproduced from Blain et al. (18) with permission.

Mitochondrial Respiration

Nonphosphorylating leak respiration rate (state 2_{CI}) and coupled respiration rates (state 3) for CI, CII, and CI þ CII, and UC_{CIV} respiration rate are summarized in Fig. 3*A*. State 2_{CI} and UC_{CIV} respiration were not different pre- and post-5 km cycling time trial in either condition. In contrast, state 3_{CI}-, state 3_{CII}-, and state $3_{CI\ þ\ CII}$-driven respiration exhibited a main effect for exercise compared with resting baseline and respiration rates were significantly decreased immediately after exercise within each condition, with the exception of state $3_{CI\ þ\ CII}$ in the FENT condition ($P = 0.08$). In terms of exercise-induced percent change in mitochondrial respiration, there was no

difference between CTRL and FENT conditions (Fig. 3*B*). The control ratios for state 2_{CI} and state $3_{CI\ þ\ CII}$ relative to UC_{CIV} are presented in Fig. 3*C*. There was no difference in state 2_{CI} control ratio pre- to postexercise; however, the state $3_{CI\ þ\ CII}$ control ratio exhibited a main effect pre- to postexercise and a significant difference within both the CTRL and FENT conditions. There was no difference in the exercise-induced percent change in state $3_{CI\ þ\ CII}$ control ratio between CTRL and FENT conditions (Fig. 3*D*). The RCR exhibited a main effect for exercise compared with resting baseline and only in the FENT condition did the decrease in RCR after exercise achieve significance (Fig. 4*A*). Furthermore, the exercise-induced percent change in RCR was significantly greater in FENT compared with CTRL (Fig. 4*B*). Finally, there was no evidence of a change in membrane integrity, assessed by the minimal change in respiration with the addition of cytochrome *c*, as a result of exercise in the CTRL or FENT conditions.

Citrate Synthase Activity

CS activity was not significantly changed from pre- to postexercise in CTRL (17 ± 12 vs. 24 ± 9 mmol/min/g, $P = 0.18$) or FENT (23 ± 8 vs. 26 ± 13 mmol/min/g, $P = 0.73$) conditions (pre vs. post, respectively).

Muscle Metabolic Perturbation and Mitochondrial Respiratory Function

Of all the negatively impacted indices of mitochondrial respiration, only the change in RCR from pre- to postexercise was significantly correlated with the degree of metabolic perturbation (Fig. 4, *C*–*E*). Specifically, the exercise-induced change in intramuscular [lactate] (Fig. 4*C*) and pH (Fig. 4*D*) were significantly related to the change in RCR ($r = –0.80$ and 0.84, respectively), whereas intramuscular Pi was not ($P = 0.56$, Fig. 4*E*). Of note, whether correlations were per- formed based on subject averages, to abide by correlation assumptions, or with each individual's multiple points, the significance of the correlations was unaffected. Therefore, for presentation purposes, each individual point has been plotted relative to the line of best fit determined from the averaged data. The reduced

"n" for Pi and lactate were the result of technical difficulties in the LC-MS analyses and, thus, the data from two subjects could not be included in the correlations. As expected, the exercise-induced changes in [lactate] and pH were significantly correlated with each other (r = -0.79).

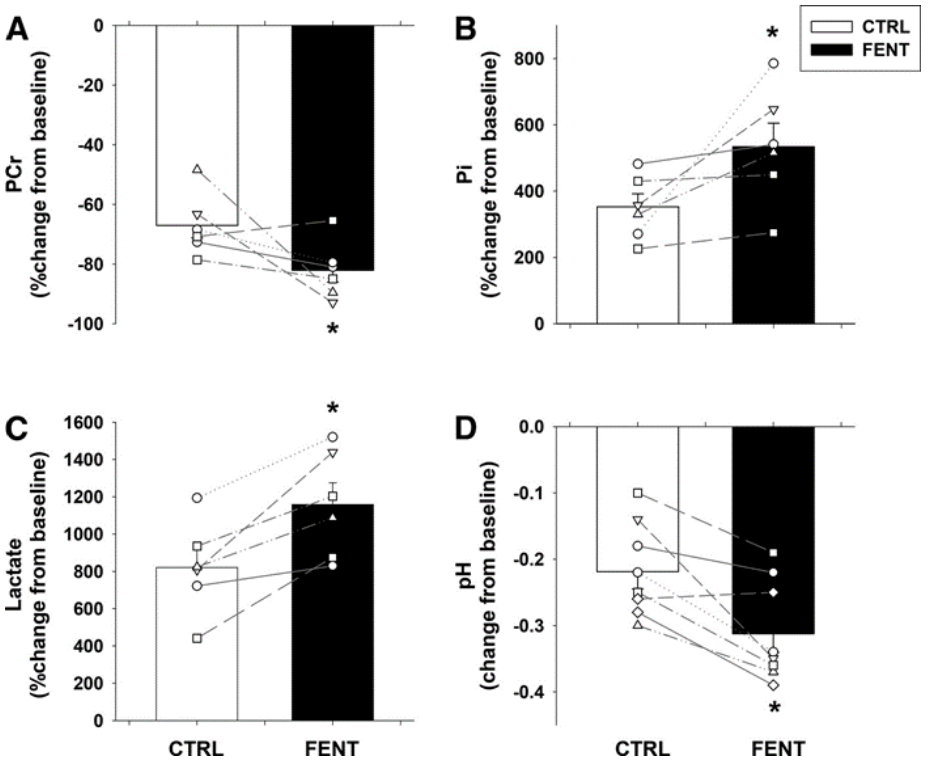

Figure 2. The change in metabolites as a consequence of a 5-km cycling time trial in control and fentanyl-treated (FENT) conditions. Samples were obtained from vastus lateralis muscle and are presented as change from pre- to postexercise. FENT resulted in greater changes in phosphocreatine (PCr) hydrolysis (*A*), inorganic phosphate (Pi) accumulation (*B*), lactate accumulation (*C*), and muscle acidity (*D*) compared with control, values are means ± SE. $^{*}P \leq 0.05$, one-tailed paired Student's *t* test, $n = 6$ subjects. Of note, the reduced "*n*" for PCr, Pi, and lactate were the result of technical difficulties in the liquid chromatography-mass spectrometer analyses and, thus, were not quantified in two subjects.

DISCUSSION

Recently it was documented that fatiguing, high-intensity exercise, resulted in a reduction in maximal mitochondrial respiratory capacity, potentially due to the metabolic perturbation associated with muscle fatigue. The present study compared mitochondrial respiratory function following high-

intensity, cycling exercise performed under both CTRL and FENT conditions to attenuate group III/IV muscle afferents and permit both an increase in muscle activation and intramuscular metabolic perturbation. Compared with CTRL, FENT resulted in a significantly greater metabolic perturbation postexercise, as documented by a greater increase in intramuscular [lactate], acidity, PCr hydrolysis, and Pi accumulation. Somewhat surprisingly, despite this greater metabolic perturbation in FENT compared with CTRL, the degree of attenuated mitochondrial respiratory capacity postexercise was not different between CTRL and FENT as assessed by maximal respiratory flux through CI, CII, CI þ CII, and state 3CI þ CII control ratio. The only exception was RCR, for which the impact of FENT was significantly greater. Although for the majority of metabolic assessments a basement effect cannot be ruled out, this fail- ure of an augmented metabolic perturbation to extensively further attenuate mitochondrial function questions the direct role of high-intensity exercise-induced metabolite accumulation in this postexercise response.

Table 2. *Summary of metabolite changes pre- to post- exercise*

	Condition	
Metabolite	**CTRL**	**FENT**
PCr	-67 ± 4%	-82 ± 4%*
Pi	353 ± 39%	-534 ± 71%*
Lactate	820 ± 101%	1,159 ± 116%*
pH	-0.22 ± 0.03	-0.31 ± 0.03*
Pyruvate	-42 ± 9%	-29 ± 14%
Cr	4 ± 15%	16 ± 6%
ATP	-21 ± 11%	-21 ± 4%
ADP	331 ± 73%	596 ± 65%*
AMP	24 ± 25%	38 ± 12%
IMP	329 ± 119%	989 ± 472%

Values are means ± SE, one-tailed paired Student's *t* test, *n* = 6–8 subjects. Data were acquired as relative changes by LC-MS (with the exception of pH measured by pH meter) since absolute could not be quantified due to a phenomenon known as ion suppression. CTRL, control; FENT, fentanyl-treated; PCr, phosphocreatine; Pi, inorganic phosphate. *$P \leq 0.05$ vs. CTRL condition.

Leg Muscle Afferent Feedback and Intramuscular Metabolic Perturbation During Exercise

Intrathecal fentanyl administration has been documented to alter perceived effort and increase muscle metabolic perturbation during both isometric (17, 39) and dynamic exercise (16, 18) by partially blocking the signal from lower limb group III/IV muscle afferents. As in the current data, such studies document greater intramuscular PCr hydrolysis, Pi accumulation, acidity, and lactate accumulation after high- intensity exercise in the FENT condition compared with CTRL despite no difference in total work (Fig. 2). Several physiological alterations occur during exercise with fentanyl blockade that contribute to this increased metabolic perturbation. Specifically, in working skeletal muscle, the greatest rate of change in intramuscular metabolites occurs at the onset of exercise (40, 41) and in the current study power out- put was elevated during the first half of the time trial in the FENT condition (Fig. 1B). This was likely as a consequence of attenuated perceived effort, with the fentanyl blockade, resulting in increased muscle activation, increased power, and an elevation in V_ O2 (Fig. 1A). The increased power in the FENT condition likely resulted in an increased metabolic perturbation at the onset of exercise that compromised cellular homeostasis throughout the exercise trial. In addition, it has been recently documented that intrathecal fentanyl increases the ATP cost of contraction during exercise (17, 39). This was characterized by Broxterman et al. (17, 39), who quantified VL muscle metabolism and ATP consumption during intermittent isometric contractions with and without fentanyl, utilizing phosphorus magnetic resonance spectroscopy. The amount of work performed between conditions was unchanged by the fentanyl treatment, but ATP consumption was increased compared with CTRL. This resulted in an increased ATP cost of contraction (DATP/N) and increased PCr hydrolysis, Pi accumulation, and muscle acidity (17, 39). Together, a transient increase in power output and an increased ATP cost of contraction as a consequence of intrathecal fentanyl likely resulted in the increased V_ O2 and augmented perturbation of cellular homeostasis during high-intensity exercise in FENT (Fig. 1 and Fig. 2).

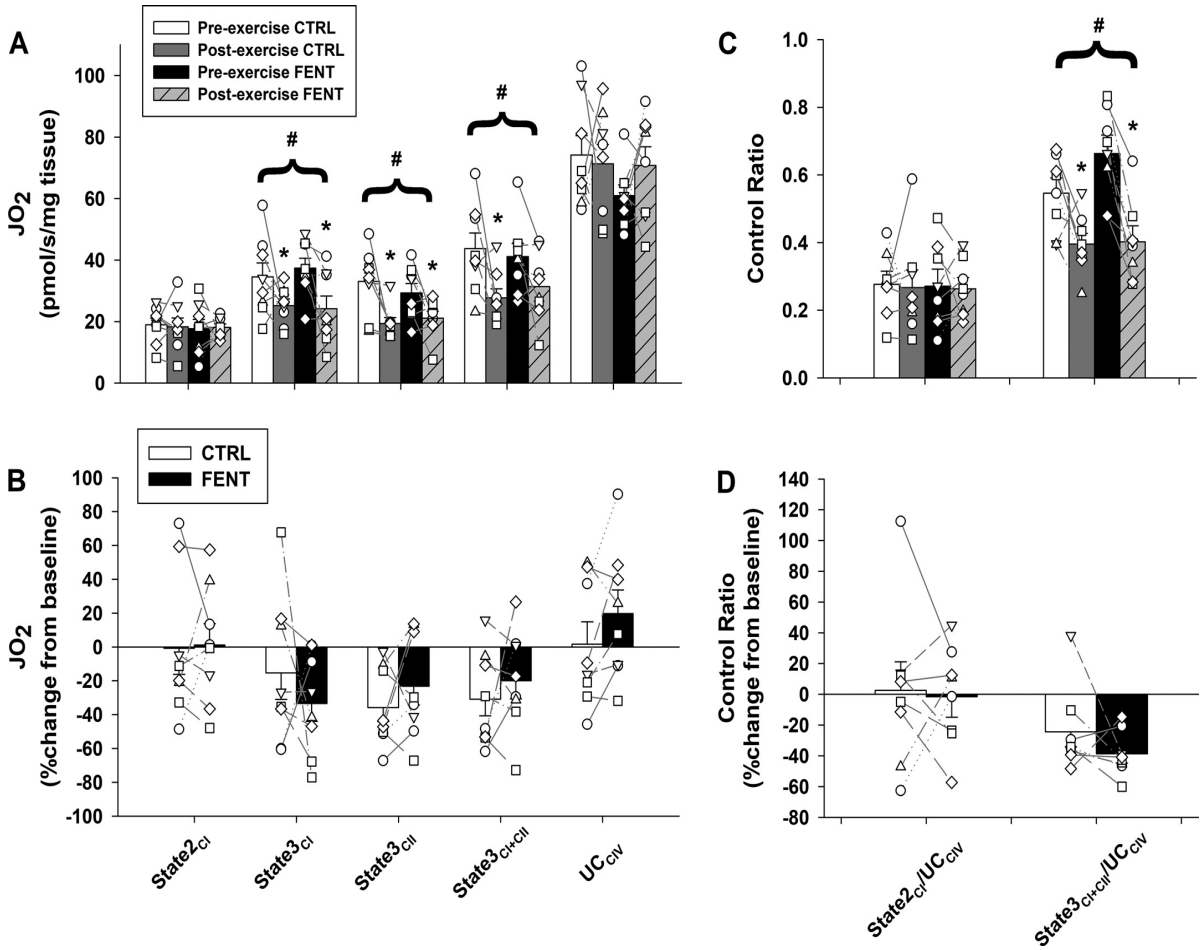

Figure 3. Mitochondrial respiratory flux assessed in permeabilized fibers from muscle samples obtained before (pre) and immediately after (post) 5-km cycling time trials in control (CTRL) and fentanyl-treated (FENT) conditions. State 3 respiratory flux was significantly attenuated pre- to postexercise (*A*), but there was no difference in the exercise-induced percent change between control and FENT conditions (*B*). State 2_{CI} control ratio, a measure of dys- coupling, showed no differences while state $3_{CI\ þ\ CII}$ control ratio, a measure of excess cytochrome *c* oxidase capacity, was significantly attenuated post- exercise (*C*), but there was no difference in the exercise-induced percent change between treatment groups (*D*). Values are means ± SE. #*P* ≤ 0.05 main effect pre- vs. postexercise, **P*≤ 0.05 vs. pre-exercise within group, two-way repeated-measures ANOVA, *n* = 8 subjects.

Exercise-Induced Intramuscular Perturbation and Mitochondrial Respiration

Recent work, by our group (1), documented impaired mitochondrial respiration, assessed in permeabilized muscle fibers, after high-intensity cycling exercise. The current study is an extension of this prior work, now documenting similar findings following exercise in both the CTRL and FENT conditions.

However, contrary to our hypotheses, de- spite greater metabolic perturbation in FENT, in general, there was little evidence of a greater impairment in mitochondrial respiration. Although a basement effect for the impact of metabolites on mitochondrial respiratory function cannot be ruled out, this failure of an augmented metabolic perturbation to extensively further attenuate mitochondrial function questions the direct role of high-intensity exercise- induced metabolite accumulation in this postexercise response. Interestingly, of the eight assessments of mitochondrial respiratory function assessed, only one, RCR, sup- ports prior studies documenting the direct impact of metabolites, namely pH, on mitochondrial respiration (Fig. 4) (10–12, 42, 43). For example, Tonkonogi and Sahlin (10) subjected isolated mitochondria to increased lactate [8 mM, within the physiological range of exercising muscle (8)] in combination with acidosis (pH = 6.38). The results documented that pH and lactate together resulted in a 47% reduction in RCR when compared with control respiration media (no lactate, pH = 7.40). In subsequent work, quantifying the sole effect of acidosis, Walsh et al. (11) documented that acidosis, alone, attenuated submaximal creatine-stimulated respiration rates in permeabilized muscle fibers.

The impact of acidosis documented in vitro has been recapitulated in vivo in cat skeletal muscle (12) and in human subjects (42, 43) during lower limb exercise, documenting reduced mitochondrial respiratory capacity in the face of acidosis. Furthermore, with respect to the degree of acidosis and the mitochondrial impact, Walter et al. (43) utilized a range of workloads to invoke progressive metabolic perturbations and quantified the rate of oxidative phosphorylation postexercise with phosphorus magnetic resonance spectroscopy. They observed a close relationship between increased acidosis and impaired mitochondrial respiration ($r = 0.79$). Although the initial observation of a negative impact of exercise-induced acidosis on mitochondrial respiration is in agreement with the current study, the overall findings contrast with this study which, with the exception of RCR, failed to document a greater metabolic impact of increased acidosis in FENT (Figs. 3 and 4). Similar work also using phosphorus magnetic resonance spectroscopy has demonstrated mitochondrial uncoupling, indicative of attenuated respiration, with increased metabolic perturbation during high-intensity

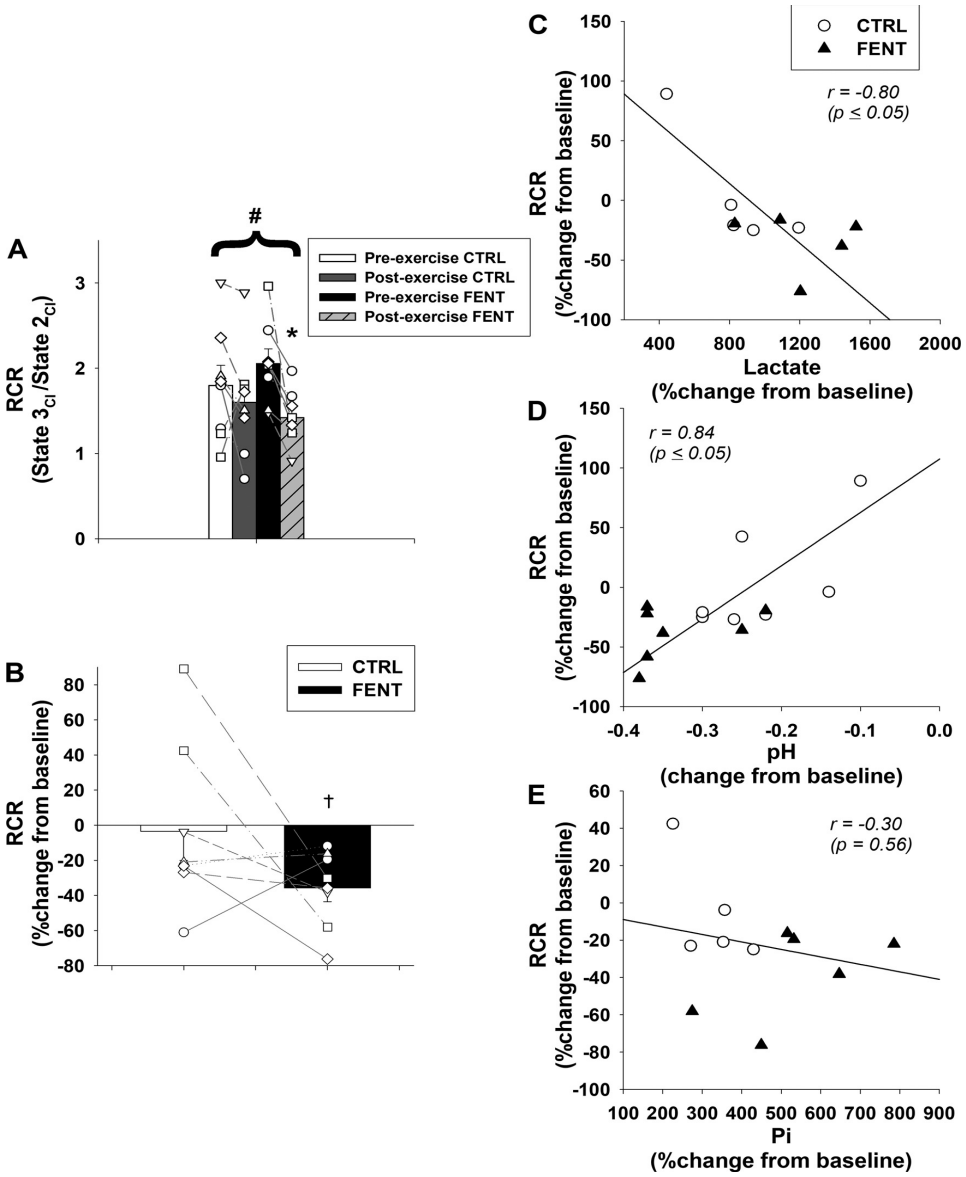

Figure 4. The respiratory control ratio (RCR) and the relationship to metabolic perturbation assessed in permeabilized fibers from muscle samples obtained before (pre) and immediately after (post) 5-km cycling time trials in control (CTRL) and fentanyl-treated (FENT) conditions. RCR was significantly attenuated postexercise (*A*), and the attenuation, expressed as exercise-induced percent change, was greater in FENT compared with CTRL (*B*). Furthermore, the exercise-induced change in RCR was significantly related to intramuscular [lactate] (*C*) and acidity (*D*), but not inorganic phosphate (Pi; *E*). Values are means ± SE (*A* and *B*) or individual subject values (*C*–*E*). #$P \leq 0.05$ main effect pre- vs. postexercise, *$P \leq 0.05$ vs. pre-exercise within group, two-way repeated-measures ANOVA, †$P \leq 0.05$, one-tailed paired Student's *t* test, $n = 6$–8 subjects in each group. Of note, the reduced "*n*" for Pi and lactate were the result of technical difficulties in the liquid chromatography-mass spectrometer analyses and, thus, the data from two subjects could not be included in the correlations.

exercise (44–46), again, in contrast with the majority of the current results (Fig. 3), with the exception of RCR (Fig. 4). Bartlett et al. (44) performed high-intensity knee-extension exercise for 24, 60, 120, and 240 s and quantified mitochondrial ATP production and ADP concentration during each challenge. Metabolic perturbation increased with exercise duration and this coincided with an attenuated rate of mitochondrial ATP production per [ADP]. Furthermore, maximal ADP-stimulated oxidative phosphorylation was reduced to an apparent basement level as there was no difference in metabolism between the 120-s and 240-s work- loads (44). These data are in direct agreement with the current finding of a reduced maximal ADP-stimulated state 3 respiration and no further attenuation with the increased metabolic perturbation in FENT for CI, CII, and CI þ CII (Fig. 3). Thus, it is possible that a basement effect for the impact of metabolites on mitochondrial respiratory function was reached in the present study.

Finally, with respect to exercise-induced intramuscular perturbation and mitochondrial respiration, a discussion of the current findings would be remiss to not, directly, address the RCR response, although an apparent outlier in this data- set. RCR is regarded as, perhaps, the most comprehensive single index of mitochondrial respiratory function as it encapsulates the most fundamental requirement for mitochondria: the ability to idle at a low rate of ATP production, yet rapidly increase this rate in response to ADP (2, 3, 30, 35, 36). Interestingly, as evidenced by RCR, there was mitochondrial uncoupling after high-intensity exercise (Fig. 4*A*), with a more definitive exercise-induced effect in the FENT condition (Fig. 4*B*). Thus, unlike other measured indices, this finding implies a greater attenuation of mitochondrial respiratory function in the presence of an increased metabolic perturbation. Supportive of this tenet, there was, in fact, a significant relationship between the exercise-induced change in RCR and both [lactate] and pH (Fig. 4, *C* and *D*). Although, as already recognized, these RCR data are in agreement with the findings of several previous studies that reported attenuated respiration with increased metabolic perturbation during high-intensity exercise (44–46), they are in the minority in the current study. Indeed, 7 of 8 metabolic assessments did not reveal a greater metabolic attenuation in FENT and the accompanying greater intramuscular perturbation.

Mitochondrial Integrity Maintained in the Face of an Increasingly Stressful Environment

Particularly novel to this study and somewhat surprisingly, despite vastly increasing the metabolic stress of the mitochondria postexercise in FENT (Fig. 2), the mitochondrial machinery appeared protected such that integrity and respiratory function were maintained relative to CTRL post- exercise (Fig. 3). This observation may be representative of "mitohormesis," defined as protective mechanisms within the integrative system that develop in response to acute stressors such as increased reactive oxygen species or metabolites (47, 48). Such mechanisms work to drive adaptations to coordinate long-term protection and ultimately improve or preserve function with a subsequent exposure (e.g., exercise training adaptations) (47, 48). Of course, if not for some such protective mechanisms, maintaining mitochondrial integrity, the required ATP production for both cell viability and avoiding rigor would become insufficient as mitochondria are repeatedly damaged.

Of note, state 2_{CI}, or basal uncoupled (leak) respiration, was unchanged pre- to postexercise (Fig. 3*A*), and similarly, cytochrome *c* tests revealed negligible responses, in both the CTRL and FENT conditions. Likewise, the maximal oxidative rate (UC_{CIV}), quantified by complete activation of cytochrome *c* oxidase activity, where oxygen is actually consumed (33, 49, 50), was unchanged (Fig. 3*A*). Together, these observations

suggest that mitochondrial membrane integrity and maximal flux, respectively, were not altered by exercise in either condition, and furthermore, are, potentially, key points of protection in high-stress conditions. Unchanged membrane integrity and maximal flux likely limits the site of impairment resulting in attenuated respiratory capacity to the phosphorylation system, supported by the observed reduction in state $3_{CI\ þ\ CII}$ control ratio following exercise in both the CTRL and FENT conditions (Fig. 3*C*) (30, 33, 35). Protection of membrane integrity and maximal flux at the expense of phosphorylative flux is consistent with the adaptive theory of mitohormesis, as the effects of high-intensity exercise are likely acute deficits in respiration (51, 52). Specifically, membrane integrity and maximal flux are essential for mitochondrial survival and subsequent regaining of function, respectively, while phosphorylative flux can be acutely impaired simply by ultrastructural changes as a result of high-intensity exercise (32, 53–55). Importantly, this may explain why prior studies which, in contrast with our previously established findings (1) and that with FENT in the current study, failed to reveal a negative impact of exercise on mitochondrial respiration (55, 56). Indeed, of note, these previous studies, showing no impact of exercise, were performed in isolated mitochondria, which do not retain the mitochondrial reticulum and, thus, the complete ultrastructure, reflective of that found in vivo, as do the permeabilized fibers employed in the current study (57–60).

Experimental Considerations

Postexercise, a cuff was used to clamp the metabolic mi- lieu before biopsies were taken, while no cuff was used for pre-exercise biopsy samples. Occlusion was necessary post- exercise to restrict metabolite recovery, which occurs with a time constant on the order of 23–29 s during free-flow conditions (61). In addition, postexercise occlusion has been documented to not increase metabolic perturbation (24–27), and, thus, the goal of clamping metabolites postexercise was likely achieved. At rest, occluding muscle blood flow likely would not impact metabolites or mitochondria collected by biopsy, as supported by a 4- to 5-min delay in metabolite accumulation from the start of occlusion (24, 28, 29, 62). Specifically, this time window is well beyond the <30 s required for biopsy sampling, and, thus, not utilizing a cuff during pre-exercise likely did not impact the results. Although unlikely, an independent effect of fentanyl administration, itself, on mitochondrial respiration cannot be ruled out. However, given the localization of fentanyl when administered within the L3-L4 region of the spinal column, fentanyl does not go systemic and, thus, it is highly unlikely that this drug could, directly, impair the mitochondria. Furthermore, any indirect impact of fentanyl on mitochondria at rest, resulting from reduced afferent activity, is also probably minimal given the already very low level of afferent activity at rest, and testing for

this alone may be gratuitous, given the subject burden involved. It is possible that cycling exercise resulted in increased intramuscular fluid, documented to increase 12%–18% during exhaustive single-leg knee extension exercise (63), potentially diluting mitochondrial content and being, falsely, interpreted as reduced respiration. However, as the permeabilized fiber technique involves washing out the cytosolic components (57), while maintaining mitochondrial/cellular structure, this methodology is not affected by changes in intramuscular fluid achieved in vivo. This tenet is supported by the lack of change in CS activity from pre- to postexercise. Finally, it must be noted that correlative significance is diminished when removing the single, near 100% change, data point from Fig. 4, *C* and *D*. The impact of a single point is inflated due to the relatively small number of sub- jects for correlative analysis, but no evidence experimentally or statistically justified this data point as an outlier, and, thus it was not removed from the analyses.

Perspectives and Significance

Administration of intrathecal fentanyl, to attenuate afferent feedback from group III/IV muscle afferents, resulted in greater intramuscular metabolic disturbance following high- intensity exercise. Somewhat surprisingly, despite this greater metabolic perturbation in FENT compared with CTRL, in general, the degree of attenuated mitochondrial respiratory function postexercise was not different between CTRL and FENT. Although a basement effect cannot be ruled out, this failure of an augmented metabolic perturbation to extensively further attenuate mitochondrial function questions the direct role of high-intensity exercise-induced metabolite accumulation in this post exercise response.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

G.M.B., M.A., and R.S.R. and conceived and designed research; G.M.B., C.R.H., G.L., M.J.R., S.-Y.P., J.D.T., J.R.G., S.K.S., J.C.W., T.J.H., J.E.J., A.D.B., M.A., and R.S.R., performed experiments; M.T.L., G.M.B., C.R.H., G.L., J.D.T., M.A., and R.S.R., analyzed data; M.T.L., G.M.B., C.R.H., G.L., M.J.R., S.-Y.P., J.D.T., J.R.G., S.K.S., J.C.W., T.J.H., J.E.J., A.D.B., M.A., and R.S.R. interpreted results of experiments; M.T.L., G.M.B., C.R.H., G.L., and R.S.R., prepared figures; M.T.L., G.M.B., C.R.H., G.L., M.J.R., S.-Y.P., J.D.T., J.R.G., S.K.S., J.C.W., T.J.H., J.E.J., A.D.B., M.A., and R.S.R. drafted manuscript; M.T.L., G.M.B., C.R.H., G.L., M.J.R., S.-Y.P., J.D.T., J.R.G., S.K.S., J.C.W., T.J.H., J.E.J., A.D.B., M.A., and R.S.R. edited and revised manuscript; M.T.L., G.M.B., C.R.H., G.L., M.J.R., S.-Y.P., J.D.T., J.R.G., S.K.S. J.C.W., T.J.H., J.E.J., A.D.B., M.A., and R.S.R. approved final version of manuscript.

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