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Consecutive bouts of diverse contractile activity alter acute responses in human skeletal muscle

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Coffey VG, Pilegaard H, Garnham AP, O'Brien BJ, and Hawley JA. Consecutive bouts of diverse contractile activity alter acute responses in human skeletal muscle. *J Appl Physiol* 106: 1187–1197, 2009. First published January 22, 2009; doi:10.1152/japplphysiol.91221.2008.—We examined acute molecular responses in skeletal muscle to divergent exercise stimuli by combining consecutive bouts of resistance and endurance exercise. Eight men [22.9 ± 6.3 yr, body mass of 73.2 ± 4.5 kg, peak O_2 uptake ($\dot{V}O_{2peak}$) of 54.0 ± 5.7 ml·kg^{-1}·min^{-1}] were randomly assigned to complete trials consisting of either resistance exercise (8 × 5 leg extension, 80% 1 repetition maximum) followed by a bout of endurance exercise (30 min cycling, 70% $\dot{V}O_{2peak}$) or vice versa. Muscle biopsies were obtained from the vastus lateralis at rest, 15 min after each exercise bout, and after 3 h of passive recovery to determine early signaling and mRNA responses. Phosphorylation of Akt and Akt1^{Ser473} were elevated 15 min after resistance exercise compared with cycling, with the greatest increase observed when resistance exercise followed cycling (~55%; $P < 0.01$). TSC2-mTOR-S6 kinase phosphorylation 15 min after each bout of exercise was similar regardless of the exercise mode. The cumulative effect of combined exercise resulted in disparate mRNA responses. IGF-I mRNA content was reduced when cycling preceded resistance exercise (−42%), whereas muscle ring finger mRNA was elevated when cycling was undertaken after resistance exercise (~52%; $P < 0.05$). The hexokinase II mRNA level was higher after resistance cycling (~45%; $P < 0.05$) than after cycling-resistance exercise, whereas modest increases in peroxisome proliferator-activated receptor gamma coactivator-1α mRNA did not reveal an order effect. We conclude that acute responses to diverse bouts of contractile activity are modified by the exercise order. Moreover, undertaking divergent exercise in close proximity influences the acute molecular profile and likely exacerbates acute "interference."

endurance; resistance exercise; acute responses; concurrent training

SKELETAL MUSCLE IS A HIGHLY malleable tissue capable of generating a multiplicity of molecular responses to divergent stimuli (12). Moreover, the plasticity of skeletal muscle enables selective attainment of the specific biochemical and physical adaptations required to attenuate disruption to cellular homeostasis with repeated overload (12). Repeated contractions of similar mode and frequency initiate adaptive responses that ultimately result in an altered phenotype. Indeed, repeated high-intensity, short-duration contraction promotes muscle hypertrophy and strength gains (20, 62), whereas prolonged, low-intensity contractile activity is associated with increased mitochondrial density and enhanced resistance to fatigue (24).

Recently, we (13, 14) and others (3, 66) have examined the specificity of acute adaptation responses to divergent contractile activity. Atherton et al. (3) reported that distinct and contrasting adaptive profiles in skeletal muscle to high-intensity, short-duration and prolonged, low-intensity contractile stimuli are incompatible: when the adenosine monophosphate-activated kinase α-peroxisome proliferator activated receptor gamma coactivator-1α (AMPK-PGC-1α) pathway was upregulated in response to an endurance stimulus, the Akt/protein kinase B-mammalian target of rapamycin-p70 S6 kinase (Akt-mTOR-S6K) pathway was repressed. Conversely, a resistance training stimulus enhanced Akt-mediated signaling while downregulating AMPK-PGC-1α activity. These results indicate that combining diverse contractile activity may not be optimal for promoting specific adaptations and that alternating the mode of contraction (termed concurrent training) may interfere with the distinct molecular profiles and likely suppress or limit the specificity of adaptive responses. We have used an experimental model in which highly trained power lifters and road cyclists performed a bout of their habitual exercise and then, on another occasion, crossed-over and undertook an unfamiliar bout of cycling or resistance exercise, respectively (13, 14). We found that chronic training history and the associated phenotype did not alter the expected adaptive profile to unfamiliar contractile activity. Therefore, the incompatibility of adaptations to resistance- or endurance-like stimuli most likely results from the interaction of the adaptive responses to each acute bout of divergent contractile activity.

The majority of research aimed at elucidating the adaptive responses to concurrent training has been confined to "end-state" measures such as maximum strength/power or maximal aerobic capacity (36, 37). With such an approach, it is impossible to deduce the timing and identity of the regulatory events that orchestrated the observed "end-point" adaptations. This lack of knowledge of the mechanisms vital to the adaptation process prevents a clear understanding of how individual contractile events interact to produce a desired adaptation. At present, there is a paucity of data pertaining to the interaction of molecular responses in human skeletal muscle when performing diverse contractile activity and little understanding of the potential mechanisms responsible for enhancing or impair-

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ing subsequent adaptation. Such information has practical relevance for understanding the mechanisms for maintaining both metabolic health and functional capacity, as well as promoting training adaptation for athletic performance. Accordingly, the aim of the present study was to examine the effect of successive bouts of resistance and endurance exercise on the early molecular responses in skeletal muscle. We hypothesized that diverse exercise undertaken in close proximity would amplify the incompatibility of the acute response to divergent contraction modes. Thus we compare the additive or interference effect when consecutive resistance and endurance bouts are undertaken in different order.

METHODS

Subjects

Eight male subjects [age 22.9 ± 6.3 yr, body mass 73.2 ± 4.5 kg, peak oxygen uptake ($\dot{V}O_{2peak}$) 54.0 ± 5.7 ml·kg^{-1}·min^{-1}, one repetition maximum leg extension (1 RM) 73.1 ± 5.3 kg; values are mean ± SD] who had been participating in regular resistance and aerobic training (>1 yr) volunteered for this study. The experimental procedures and possible risks associated with the study were explained to each subject, who gave written, informed consent before participation. The study was approved by the Human Research Ethics Committee of RMIT University.

Study Design

The study employed a randomized crossover design where each subject completed two experimental training sessions separated by 2 wk. One experimental trial consisted of a bout of resistance exercise followed by a bout of endurance exercise (cycling), whereas in the other trial subjects performed the reverse exercise order (i.e., endurance and then resistance exercise; Fig. 1).

Preliminary Testing

$\dot{V}O_{2peak}$. $\dot{V}O_{2peak}$ was determined during an incremental test to exhaustion on a Lode cycle ergometer (Groningen, The Netherlands). The protocol has been described in detail previously (25). In brief, subjects commenced cycling at a workload equivalent to 2 W/kg for 150 s. Thereafter, the workload was increased by 25 W every 150 s until volitional fatigue, defined as the inability to maintain a cadence >70 revolutions/min. Throughout the test, which typically lasted 12–14 min, subjects breathed through a mouthpiece attached to a metabolic cart (Parvomedics, Sandy, UT) to record oxygen consumption.

Maximal strength. Quadriceps strength was determined during a series of single repetitions on a plate-loaded leg extension machine until the maximum load lifted was established (1 RM). Repetitions were separated by a 3-min recovery and were used to establish the maximum load/weight that could be moved through the full range of motion once, but not a second time. Exercise range of motion was 85°, with leg extension endpoint set at −5° from full extension.

Diet/Exercise Control

Before both experimental trials (described below), subjects were instructed to refrain from training and other vigorous physical activity for a minimum of 48 h. Subjects were provided with standardized prepacked meals that consisted of 3 g carbohydrate/kg body mass, 0.5 g protein/kg body mass, and 0.3 g fat/kg body mass consumed as the final caloric intake the evening before reporting for an experimental trial.

Experimental Trials

An overview of the study protocol is shown in Fig. 1. On the morning of an experimental trial, subjects reported to the laboratory after an ~10-h overnight fast. After subjects rested in the supine position for ~15 min, local anesthesia [2–3 ml of 1% Xylocaine (lignocaine)] was administered to the skin, subcutaneous tissue, and fascia of the vastus lateralis of the subject's leg in preparation for the series of muscle biopsies. A resting (basal) biopsy was taken using a 5-mm Bergstrom needle modified with suction. Approximately 150 mg of muscle was removed, blotted to remove excess blood, and immediately frozen in liquid nitrogen. Subjects then completed the two exercise sessions (described in detail subsequently). Fifteen minutes after completion of the first exercise bout, a second biopsy was taken. Subjects then performed the second exercise bout. After a 15-min recovery from the second exercise session, a third muscle biopsy was taken and subjects rested in the supine position until a fourth muscle biopsy was taken after the 3-h recovery period. Each muscle biopsy was taken from a separate site distal to proximal from the right leg for the first trial and left leg for the second trial with all samples stored at −80°C until subsequent analysis. In addition, blood samples (~2 ml) were taken at rest preexercise, during (15 min) each exercise bout, and immediately and 15 min after each exercise bout.

Resistance Exercise

After a standardized warm-up (2 × 5 repetitions at 50% and 60% 1 RM, respectively), subjects performed eight sets of five repetitions at ~80% 1 RM. Each set was separated by a 3-min recovery period during which the subject remained seated on the leg extension machine. Contractions were performed at a set metronome cadence approximately equal to 30°/s, and strong verbal encouragement was provided during each set.

Cycling Exercise

Subjects performed 30 min of continuous cycling at a power output that elicited ~70% of individual $\dot{V}O_{2peak}$. Subjects were fan-cooled and allowed ad libitum access to water throughout the ride. Visual feedback for pedal frequency, power output, and elapsed time were provided to subjects.

Analytical Procedures

Blood glucose and lactate and muscle glycogen. Whole blood was immediately analyzed for glucose and lactate concentration using an automated glucose/lactate analyzer (YSI 2300, Yellow Springs, OH).

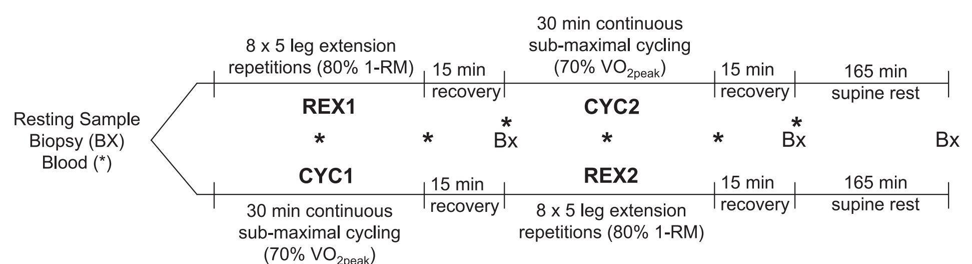

Fig. 1. Schematic of the experimental trials incorporating consecutive bouts of exercise. The study utilized a randomized cross-over design, which subjects completed for both exercise orders. Subjects undertook passive recovery (rest) between each exercise bout and throughout the 3-h recovery period. CYC, cycling; REX, resistance exercise; 1 RM, one repetition maximum; $\dot{V}O_{2peak}$, peak O_2 uptake.

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A small piece of frozen muscle (~20 mg) was freeze-dried and powdered to determine muscle glycogen concentration (39). Freeze-dried muscle was extracted with 500 μl of 2 M hydrochloric acid, incubated at 100°C for 2 h, and then neutralized with 1.5 ml of 0.667 M sodium hydroxide for subsequent determination of glycogen concentration via enzymatic analysis with fluorometric detection (Jasco FP-750 spectrofluorometer, Easton, MD). Glycogen concentration was expressed as millimoles of glycogen per kilogram of dry weight.

Western blots. Muscle samples were homogenized in buffer containing 50 mM Tris·HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM DTT, 10 μg/ml trypsin inhibitor, 2 μg/ml aprotinin, 1 mM benzamidine, and 1 mM PMSF. After determination of protein concentration (Pierce, Rockford, IL), lysate was resuspended in Laemmli sample buffer, separated by SDS-PAGE, and transferred to polyvinylidine fluoride membranes blocked with 5% non-fat milk, washed with 10 mM Tris·HCl, 100 mM NaCl, and 0.02% Tween 20, and incubated with primary antibody (1:1,000) overnight at 4°C. Membranes were incubated with secondary antibody (1:2,000), and proteins were detected via chemiluminescence (Amersham Biosciences, Buckinghamshire, UK; Pierce Biotechnology, Rockford, IL) and quantified by densitometry. All sample time points for each subject were run on the same gel. Polyclonal anti-phospho-mTOR^{Ser2448} (no. 2971), -p70 S6K$^{Thr421/Ser424}$ (no. 9204), monoclonal anti-phospho-Akt^{Ser473} (no. 9271), -tuberin (TSC2)^{Thr1462} (no. 3617), -p70 S6K^{Thr389} (no. 9206), -S6 ribosomal protein$^{Ser235/6}$ (no. 4856), anti-Akt (no. 9272), -TSC2 (no. 3635), −p70 S6K (no. 9202), -S6 ribosomal protein (no. 2217), and -adenosine monophosphate kinase-α (AMPKα) (no. 2603) were from Cell Signaling Technology (Danvers, MA). Monoclonal anti-phospho-Akt1^{Ser473} (no. 05-669) and anti-mTOR (no. 07-231) were from Upstate Cell Signaling Solutions (Temecula, CA). Anti-phospho-AMPKα^{Thr172} was raised against AMPK-α peptide (KDGEFLRpTSCGAPNY) as described previously (11).

RNA isolations, reverse transcription, and PCR. Total RNA was isolated from ~25 mg of muscle tissue, and the final RNA pellet was resuspended in 1 μl/mg original tissue in diethyl pyrocarbonate-treated H_2O containing 0.1 mM EDTA as previously described (50). Reverse transcription was performed on 3 μg of total RNA of each sample using the Superscript II RNase H^- system with oligo(dT) (Invitrogen, Carlsbad, CA), and the RT products were diluted in nuclease-free H_2O as previously described (50). The amount of single-stranded DNA was determined in the RT samples using OliGreen reagent (Molecular Probes) as previously described (40). The mRNA content was determined for selected genes using fluorescence-based real-time PCR (ABI PRISM 7900 sequence detection system, Applied Biosystems). Forward primers and reverse primers and TaqMan probes were designed from human-specific sequence data (Entrez-NIH and Ensembl, Sanger Institute) using computer software (Primer Express, Applied Biosystems). The primer and probe sequences are given in Table 1. The probes were 5′,6-carboxyfluorescein and 3′,6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine labeled. Prior optimization was performed to determine optimal primer concentrations and probe concentration and to verify the efficiency of the amplification. PCR amplification was performed in triplicate in a total reaction volume of 10 μl with 21 ng cDNA as previously described (40). Serial dilutions were made from a pooled representative sample, and these samples were amplified together with the unknown samples and used to construct a standard curve. The obtained critical threshold values reflecting the initial content of the specific transcript in the samples were converted to an arbitrary amount by using the standard curve. For each sample, the amount of a given target cDNA was normalized to the single-stranded DNA content in the sample.

Statistical Analysis

All data were analyzed by two-way repeated-measures ANOVA (two factor: time × exercise order) with Student-Newman-Kuels post hoc analysis. Statistical significance was established when $P < 0.05$ (SigmaStat for windows Version 3.11). Lacking any information on the smallest substantial changes in acute responses to divergent exercise, we performed additional post hoc analysis in which data of each individual were back transformed against the mean of their corresponding resting preexercise values. For detailed statistical methods employed in the present study, the reader is referred to Hopkins et al. (27). Briefly, log-transformed delta values between data time points were directly compared and converted to Cohen effect sizes (ES) to determine the magnitude of change for each exercise bout and alternate exercise order (26). We chose a default confidence interval of 90% to calculate ES via a spreadsheet making the same assumptions about sampling distributions that statistical packages use to derive *P* values (26). We interpreted the magnitude of the ES by using conventional threshold values of 0.2 as the smallest effect, 0.5 as a moderate effect, and 0.8 as a large ES (15, 26). Data are expressed as arbitrary units ± SD.

RESULTS

Blood Lactate and Glucose

Blood lactate concentration was significantly different over time but not exercise order ($P < 0.001$; Table 2). Blood lactate concentrations were significantly elevated above rest during and immediately postexercise after both exercise bouts independent of order ($P < 0.05$). There were no significant changes in blood glucose concentration over the experimental period (Table 2).

Muscle Glycogen

There were significant differences in muscle glycogen concentration for time and exercise order ($P < 0.01$; Table 2). There were similar decreases in muscle glycogen concentration relative to rest during each cycling bout regardless of exercise

Table 1. *Primer/probe sequences used for real-time PCR*

Gene	Forward Primer	Reverse Primer	Taqman Probe
HKII	5′-TTGTCCGTAACATTCTCATCGATT-3′	5′-TGTCTTGAGCCGCTCTGAGAT-3′	5′-ACCAAGCGTGGACTGCTCTTCCGA-3′
PGC-1α	5′-CAAGCCAAACCAACAACTTTATCTCT-3′	5′-CACACTTAAGGTGCGTTCAATAGTC-3′	5′-AGTCACCAAATGACCCCAAGGGTTCC-3′
PGC-1β	5′-GAGGGCTCCGGCACTTCT-3′	5′-CATGGCTTCATACTTGCTTTTCC-3′	5′-CCCAGATACACTGACTACGATTCCAATTCAGAAG-3′
Atrogin	5′-GATGTTACCCAAGGAAAGAGCAGTAT-3′	5′-ACGGATGGTCAGTGCCCTT-3′	5′-CCCTTCAGCTCTGCAAACACTGTCACAT-3′
MuRF	5′-GGAGCCACCTTCCTCTTGACT-3′	5′-CTCAAAGCCCTGCTCTGTCTTC-3′	5′-AACTCATCAAAAGCATTGTGGAAGCTTCCAA-3′
IGF-IEa	5′-CAGCGCCACACCGACAT-3′	5′-TTGTTTCCTGCACTCCCTCTACT-3′	5′-AAGACCCAGAAGGAAGTACATTTGAAGAACGC-3′
MGF	5′-ACGAAGTCTCAGAGAAGGAAAGGA-3′	5′-CGGTGGCATGTCACTCTTCA-3′	5′-ACATTTGAAGAACGCAAGTAGAGGGAGTGCA-3′
MyoD	5′-TGCCACAACGGACGACTTC-3′	5′-CGGGTCCAGGTCTTCGAA-3′	5′-CCGTGTTTCGACTCCCCGGACCT-3′

HKII, hexokinase II; PGC-1α, peroxisome proliferator activated receptor gamma co-activator 1α; PGC-1β, peroxisome proliferator activated receptor gamma co-activator 1β; MuRF, muscle ring finger; MGF, mechano-growth factor; MyoD, myogenic differentiation factor.

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Table 2. *Blood lactate and glucose concentration and muscle glycogen concentration measured during each training session incorporating alternate exercise orders*

	REX1				CYC2		
	Rest	15 min	PostEx	15 min Post1	15 min	PostEx	15 min Post2
Lactate, mmol/l	0.81±0.3	3.70±1.0*†‡	4.48±0.9*†‡	2.07±0.7*	3.66±1.1*†‡	3.31±0.7*†‡	1.10±0.5
Glucose, mmol/l	4.56±0.3	4.25±0.8	4.74±0.4	4.54±0.1	4.09±0.3	4.11±0.9	4.38±0.2
Glycogen, mmol/kg dry wt	456±114			412±99			218±89*†
	CYC1				REX2		
	Rest	15 min	PostEx	15 min Post1	15 min	PostEx	15 min Post2
Lactate, mmol/l	0.78±0.4	3.15±1.2*†‡	2.97±1.3*	1.56±0.5	3.31±1.6*†‡	3.62±1.7*†‡	1.31±0.4
Glucose, mmol/l	4.45±0.2	4.01±0.3	4.45±0.4	4.39±0.2	4.16±0.8	4.34±0.2	4.48±0.2
Glycogen, mmol/kg dry wt	430±109			251±82*			217±109*

Values are means ± SD. PostEx, postexercise. Significantly different ($P < 0.05$) versus *rest, †15 min postexercise 1 (15 min Post1); ‡15 min postexercise 2 (15 min Post2).

order [cycle bout 2 (CYC2) ~40% vs. cycle bout 1 (CYC1) ~42%, $P < 0.001$; Table 2]. Likewise, glycogen utilization during each resistance exercise bout was comparable [resistance exercise bout 1 (REX1) ~12% vs. resistance exercise bout 2 (REX2) ~14%], but there was an order effect for resistance exercise (REX1 vs. REX2, $P = 0.009$) due to a significant difference from resting glycogen concentration only when cycling preceded resistance exercise (CYC1-REX2, $P < 0.001$; Table 2). There were no differences in total muscle glycogen utilization between the experimental trials (Table 2).

Signaling Responses

Akt-TSC2-mTOR. There were significant changes in Akt^{Ser473} phosphorylation with time in both REX1-CYC2 and CYC1-REX2 exercise orders ($P < 0.001$; Fig. 2*A*). There was a disparity in Akt phosphorylation 15 min after the initial bout of resistance exercise (REX1) compared with 30 min of cycling (CYC1, ~120% ES = 1.1). However, an initial bout of cycling did not prevent an exercise-induced increase in phosphorylation from rest when a subsequent bout of resistance exercise was performed (REX2, $P = 0.002$), and REX2 resulted in a greater change from rest than REX1 (~55%, ES = 0.8; Fig. 2*A*). Akt phosphorylation after CYC2 remained elevated but was not additive when undertaken after resistance training (CYC2 vs. CYC1, ~64%, ES = 0.7). Phosphorylation of Akt returned to resting levels 3 h after cessation of contractile activity regardless of exercise order. In addition, phosphorylation of the AKT1 isoform at Ser473 was highly coordinated with that of Akt, resulting in significant differences at equivalent time points with larger ES (REX1 vs. CYC1, ~178%, ES = 1.4; CYC2 vs. CYC1, ~106%, ES = 1.0; Fig. 2*B*).

Significant main effects in tuberin (TSC2)^{Thr1462} phosphorylation were observed for time ($P = 0.007$), and there was also a significant difference within resistance exercise ($P = 0.04$; Fig. 2*C*). Phospho-TSC2 was increased above resting levels 3 h after both exercise orders and was significantly different for REX1-CYC2 ($P = 0.03$) but not for CYC1-REX2 ($P = 0.056$). There were also small-to-moderate increases in the acute signaling responses 15 min after exercise, with the largest changes in TSC2 phosphorylation after the first bout regardless of exercise mode (REX1 vs. REX2, ~33%, ES = 0.5; CYC1 vs. CYC2, ~45%, ES = 0.7). Of note, there was a disparity between TSC2 phosphorylation 3 h after completion of exercise with a higher value from rest after REX1-CYC2 than after CYC1-REX2 (~55%, ES = 1.0).

Changes in mTOR^{Ser2448} phosphorylation resulted in significant time × exercise order interactions (Fig. 2*D*). Moreover, phosphorylation of mTOR was different between REX1 vs. REX2 ($P = 0.05$, ES = 1.1) and CYC1 vs. CYC2 ($P = 0.04$, ES = 1.9). However, the decreased phosphorylation state 15 min after a second consecutive exercise bout compared with the initial response was not significantly different (Fig. 2*D*). A significant decrease in phospho-mTOR was observed after 3 h of recovery in the REX1-CYC2 trial but not the reverse exercise order (−61%, $P = 0.025$, ES = 0.8).

p70 S6K-ribosomal S6 protein-AMPK. Changes in p70 S6K at Thr421/Ser424 failed to reach statistical significance despite the substantial increases in phosphorylation after an initial bout of exercise ($P = 0.08$; Fig. 3*A*). Moreover, there was little variation in the phosphorylation response to the initial bouts of divergent contractile activity (REX1 vs. CYC1, ~25%, ES < 0.2). A decrease in p70 S6K$^{Thr421/Ser424}$ phosphorylation was observed after each successive bout of contractile overload regardless of exercise mode (−75%, ES = 0.8; Fig. 3*A*). Increased phosphorylation was moderately higher 3 h after REX1-CYC2 than after CYC1-REX2 compared with rest (~60%, ES = 0.5). Changes in p70 S6K^{Thr389} corresponded with Thr421/Ser424, and there were significant differences for time ($P < 0.001$; Fig. 3*A*). The increase in p70 S6K^{Thr389} phosphorylation with the initial exercise bouts was significantly elevated above rest after cycling (CYC1, $P = 0.01$) but not resistance exercise (REX1, $P = 0.06$; Fig. 3*B*). Moreover, there were no differences in the magnitude of the increase in p70 S6K phosphorylation from rest between the divergent exercise modes (ES = 0.06). However, after each successive exercise bout, the contraction-induced increase in phosphorylation at Thr389 was sustained with REX1-CYC2 ($P = 0.03$) but abated after CYC1-REX2 (−46%, ES = 1.0). After 3 h of recovery, p70 S6K phosphorylation was moderately higher after REX1-CYC2 than after CYC1-REX2, but this difference was not statistically significant (33%, ES = 0.65).

There was a significant interaction between time and exercise order for S6 ribosomal protein Ser235/6 phosphorylation ($P = 0.01$). The comparable increases in S6 phosphorylation

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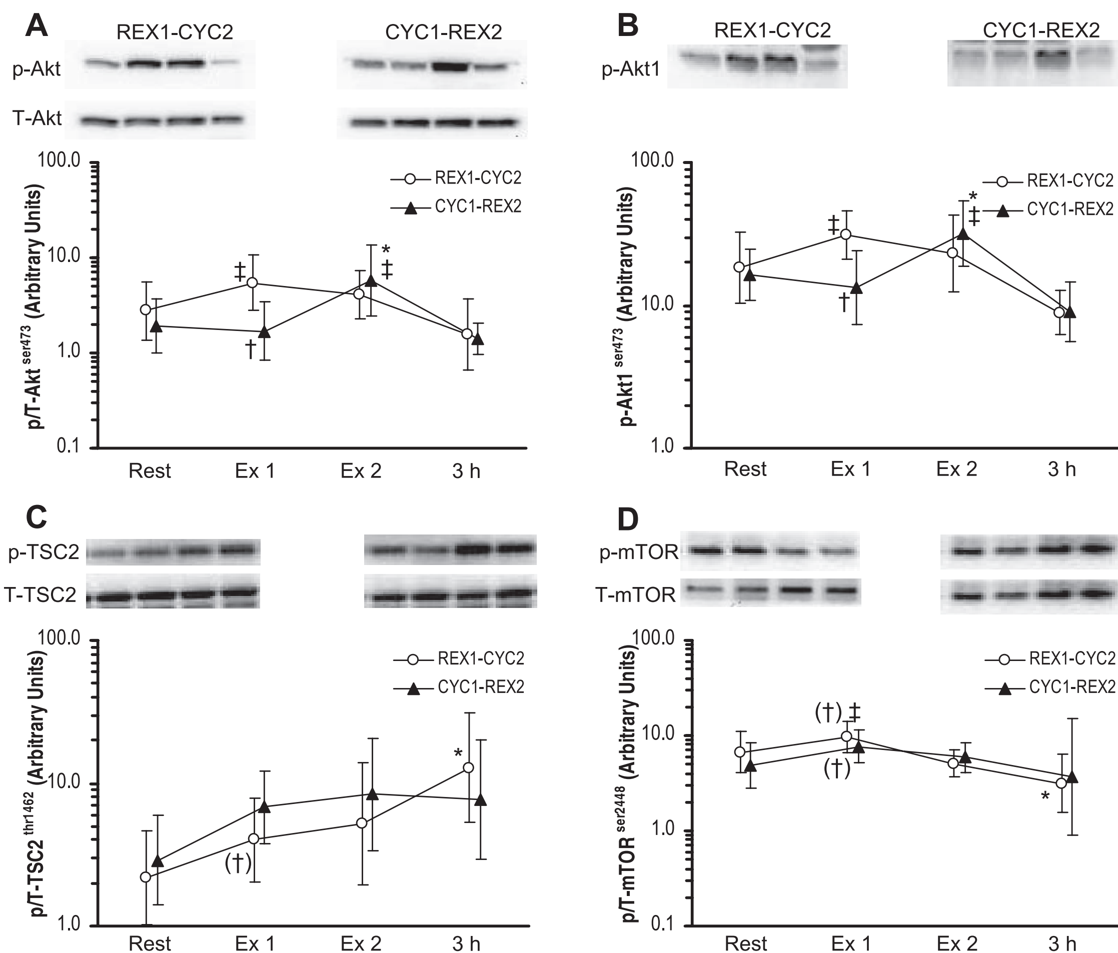

Fig. 2. Phosphorylated Akt^{Ser473} (*A*), isoform-specific Akt1^{Ser473} (*B*), tuberin^{Thr1462} (TSC2; *C*), and mammalian target of rapamycin^{Ser2448} (mTOR; *D*) relative to total protein at rest preexercise, 15 min after each individual exercise bout (Ex 1 and Ex 2), and 3 h after cessation of the second exercise bout. Subjects completed 2 experimental trials incorporating consecutive resistance exercise (8 × 5 leg extensions at 80% 1 RM) and cycling (30 min at ~70% $\dot{V}O_{2peak}$) bouts performed in alternate order. Results are group means (± SD), and data are log-transformed values as arbitrary units. Significant difference ($P < 0.05$) vs. *rest, †Ex 2, (†)alternate Ex 2, ‡3 h.

after the initial exercise bout were different from rest after resistance exercise (REX1, $P = 0.049$) but not cycling (CYC1, $P = 0.08$; Fig. 3*C*). However, the delta increase from rest was greater after the initial bout of cycling than after resistance exercise (~65%, ES = 0.4). Furthermore, the enhanced phosphorylation at Ser235/6 after REX1 was higher than 15 min after REX2 ($P = 0.01$), and 3 h after cessation of contractile activity, S6 phosphorylation was significantly elevated after CYC1-REX2 compared with REX1-CYC2 ($P = 0.01$, ES 0.8).

There were no differences in AMPK^{Thr172} phosphorylation 15 min after resistance or cycling exercise (Fig. 3*D*). Phosphorylation of AMPK 3 h postexercise was not significantly different from rest for any exercise order but was higher after REX1-CYC2 than after CYC1-REX2 (~45%, ES = 0.9).

mRNA Responses

IGF-IEa-mechano-growth factor-myogenic differentiation factor. Changes in IGF-IEa and mechano-growth factor [MGF (IGF-IEc)] mRNA abundance 3 h after exercise were not significantly different from rest. However, the increase in IGF-IEa mRNA was attenuated when cycling preceded resistance exercise (CYC1-REX2, −42%, ES = 0.4; Fig. 4*A*), and there was also a decrease in MGF mRNA when cycling was undertaken first (CYC1-REX2, −27%, ES = 0.3; data not shown). There was an exercise-order effect in myogenic differentiation factor (MyoD) mRNA abundance within rest ($P = 0.009$) and 3 h postexercise ($P = 0.048$; Fig. 4*B*). Exercise did not promote a significant increase in MyoD after the 3-h recovery and resulted in no difference in ES (ES = 0.06).

Atrogin-muscle ring finger. The atrogin mRNA content increased after consecutive exercise bouts but was not significantly different from rest for any exercise order (ES = 0.14, Fig. 4*C*). There was a significant increase in muscle ring finger (MuRF) mRNA abundance from resting values when resistance exercise preceded cycling (REX1-CYC2, $P = 0.009$) but not after the reverse exercise order (CYC1-REX2, $P = 0.3$; Fig. 4*D*). Moreover, changes in delta mRNA abundance indicate that MuRF transcriptional activity was exacerbated when cycling was undertaken after resistance exercise (REX1-CYC2 vs. CYC1-REX2, ~52%, ES = 0.4).

PGC-1α/β-hexokinase II. There was a significant effect among the different levels of time (rest vs. 3 h, $P = 0.024$) for PGC-1α, but these differences were not significant within each individual exercise order (REX1-CYC2, $P = 0.17$, CYC1-

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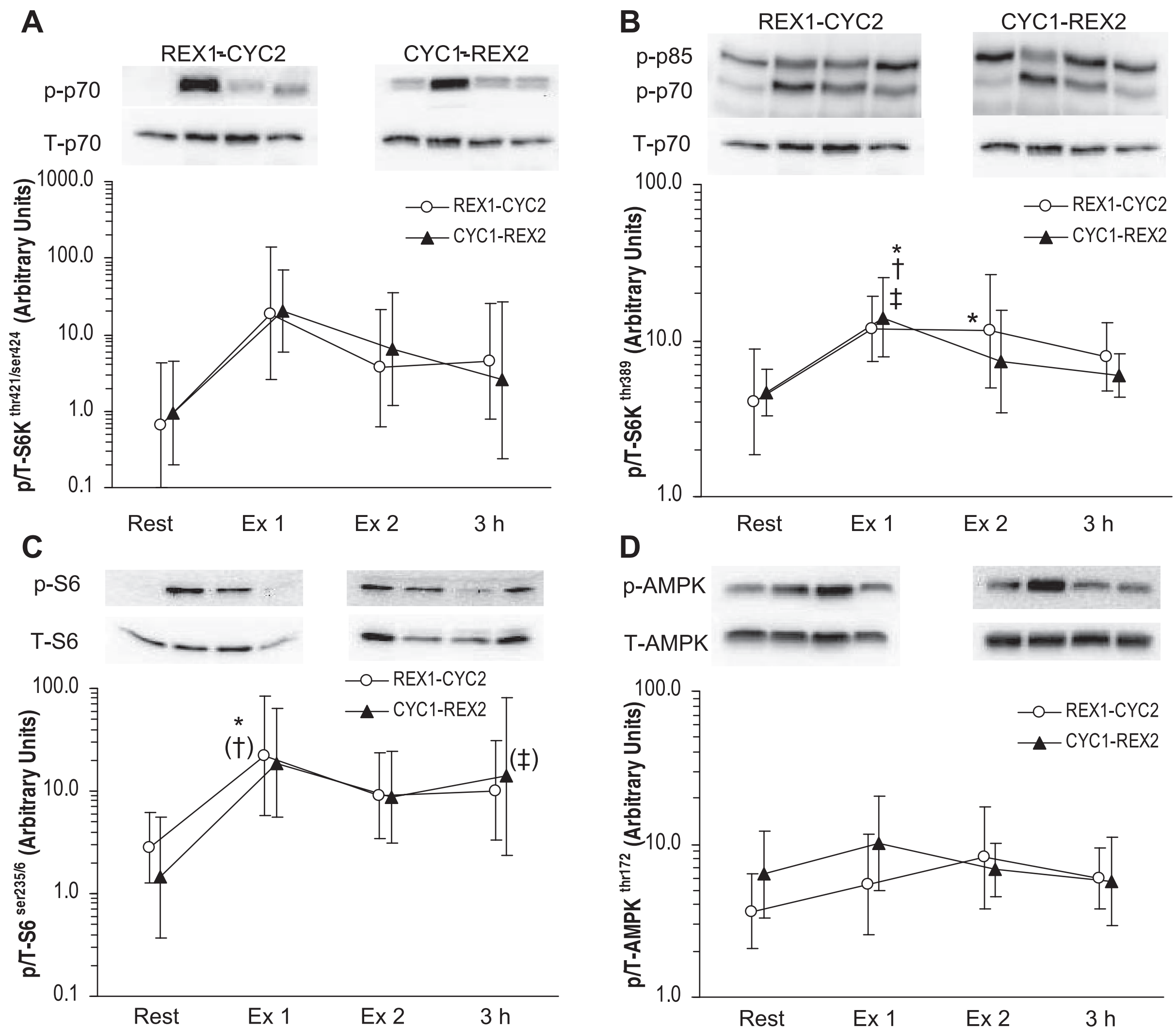

Fig. 3. Phosphorylated p70 S6 kinase$^{Thr421/Ser424}$ (p70 S6K; *A*), p70 S6K^{Thr389} (*B*), S6 ribosomal protein$^{Ser235/6}$ (S6; *C*), and adenosine monophosphate activated kinase-α^{Thr172} (AMPK; *D*) relative to total protein at rest preexercise, 15 min after each individual exercise bout (Ex 1 and 2), and 3 h after cessation of the second exercise bout. Subjects completed 2 experimental trials incorporating consecutive resistance exercise (8 × 5 leg extensions at 80% 1 RM) and cycling (30 min at ~70% $\dot{V}O_{2peak}$) bouts performed in alternate order. Results are group means (±SD), and data are log-transformed values as arbitrary units. Significant difference ($P < 0.05$) vs. *rest, †Ex 2, (†)alternate Ex 2, ‡3 h, and (‡)alternate 3 h.

REX2, $P = 0.07$; Fig. 5*A*). Indeed, despite a small positive effect when cycling was undertaken before resistance exercise, there was little difference in ES between the alternate exercise orders (CYC1-REX2 vs. REX1-CYC2, ~46%, ES = 0.2) Similarly, there were no significant changes in PGC-1β mRNA with a small (~40%) elevation in mRNA when cycling exercise followed resistance exercise (REX1-CYC2, ES = 0.3; Fig. 5*B*). Hexokinase II (HKII) mRNA abundance increased significantly from rest following the resistance-cycling exercise order ($P = 0.006$), and there was only a small positive effect when cycling preceded resistance exercise compared with cycling after resistance exercise (~45%, ES = 0.3; Fig. 5*C*).

DISCUSSION

Skeletal muscle adaptation after repeated bouts of contractile activity is highly specific to the overload stimulus (3), with the specificity of adaptation easily identified by comparing the divergent phenotypes of endurance- vs. resistance-trained athletes (17, 20). In the present study, we have utilized consecutive high-intensity intermittent (resistance) and continuous, moderate-intensity (endurance) contractions in humans to determine the specificity of acute adaptive responses and potential additive or interference effects with divergent stimuli. The results from the present investigation provide novel evidence of altered cell signaling and mRNA responses in an exercise order-dependent manner in skeletal muscle. Specifically, we show comparable insulin/IGF pathway signaling downstream of Akt during the early recovery phase (15 min postexercise) regardless of the mode of contraction and divergence in the specificity of acute responses for individual protein and mRNA markers after the 3-h recovery.

We employed a crossover design whereby recreationally trained subjects undertook two experimental trials under the same conditions comprising consecutive endurance and resistance exercise bouts in which the cumulative overload stimulus was similar for both trials but the exercise order was different (Fig. 1). In this regard, blood lactate and glucose responses to the divergent contractile activity were similar, as was total

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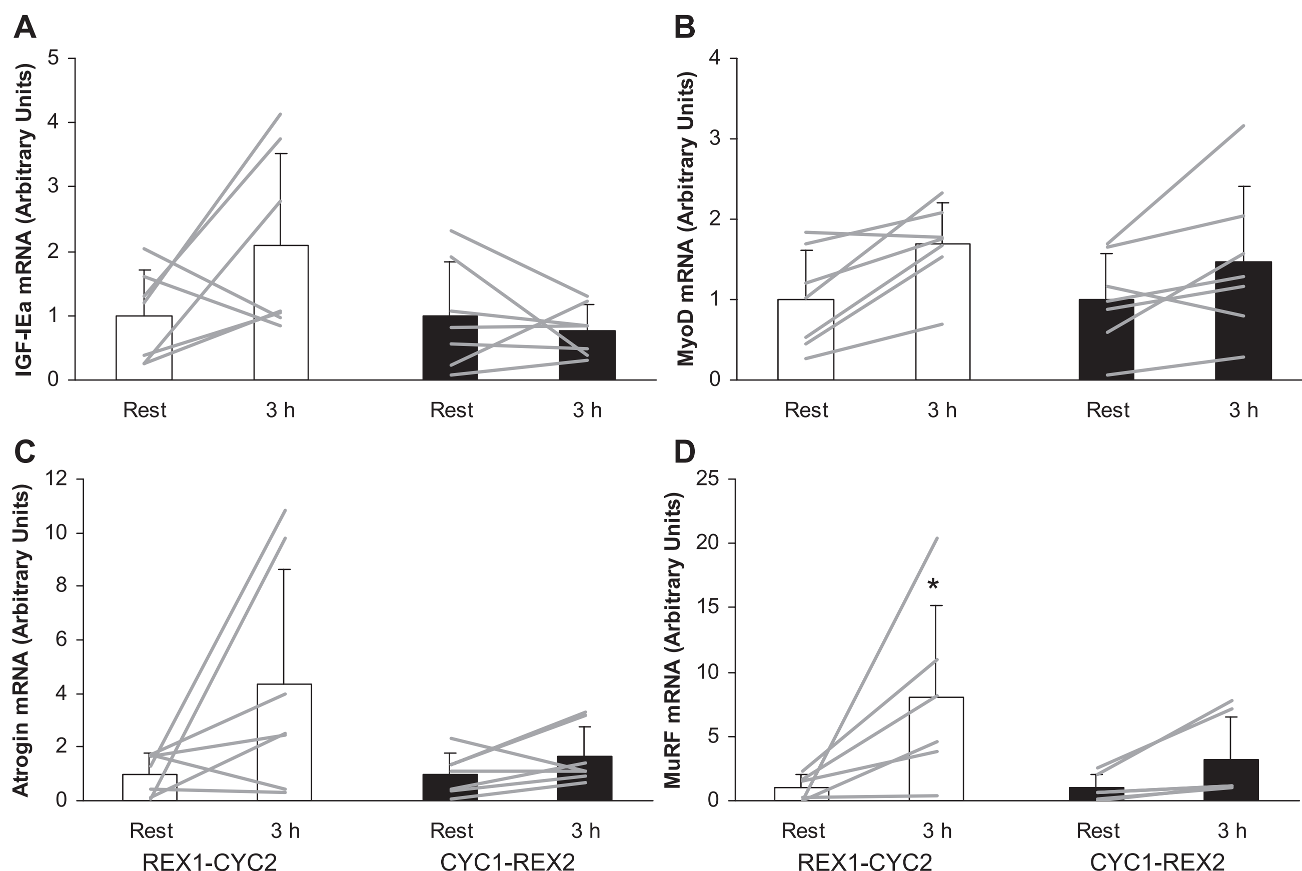

Fig. 4. Isoform-specific IGF-IEa (*A*), myogenic differentiation factor (MyoD; *B*), atrogin (*C*), and muscle ring finger (MuRF; *D*) mRNA abundance at rest preexercise and 3 h after cessation of the second exercise bout. Subjects completed 2 experimental trials incorporating consecutive resistance exercise (8 × 5 leg extensions at 80% 1 RM) and cycling (CYC; 30 min at ~70% $\dot{V}O_{2peak}$) bouts performed in alternate order. Results are individual responses and group means (±SD) in arbitrary units. Significant difference ($P < 0.05$) vs. *rest.

glycogen utilization during the two experimental trials (Table 2). AMPK has been termed a metabolic "master controller" activated by exercise and changes in glycogen content in skeletal muscle (31). AMPK phosphorylation was not different 15 min after each successive exercise bout (Fig. 3*D*). However, phosphorylation of AMPK above rest was higher 3 h after cycling was undertaken after resistance exercise, indicating metabolic stress may have been exacerbated when endurance exercise was performed subsequent to resistance exercise.

A novel finding of the present study was the divergence in Akt^{Ser473} phosphorylation 15 min after resistance compared with endurance exercise (Fig. 2*A*). Moreover, the increases in isoform-specific Akt1 phosphorylation with resistance but not endurance exercise is indicative of the capacity for high-intensity, low-volume contraction to promote an anabolic response in skeletal muscle (3, 9) (Fig. 2*B*). Notably, a prior bout of cycling did not prevent an increase in Akt phosphorylation with subsequent resistance exercise, whereas a bout of cycling did not augment phosphorylation of Akt. However, we cannot rule out the possibility that the preceding endurance exercise bout was additive to Akt phosphorylation 15 min after subsequent resistance exercise, as a greater acute response was seen when resistance exercise followed cycling. Previous studies reveal a range of Akt phosphorylation responses to contractile activity in human skeletal muscle (12). Moreover, Akt phosphorylation has been shown to increase after both resistance (16, 18) and endurance activities (14, 28, 41, 65) or to remain unchanged with exercise (14, 19, 42, 61, 63). In the present investigation, we observed increased Akt phosphorylation after resistance exercise, with the greatest magnitude of change when resistance exercise was undertaken after cycling. The possibility exists that 30 min of moderate-intensity cycling is insufficient to alter Akt activity or that diverse contractile overload results in altered time-course activation. Nonetheless, although cycling did not increase Akt phosphorylation, it seems reasonable to suggest that endurance-like contractile activity does not inhibit the Akt response to resistance training.

Subtle changes in TSC2 and mTOR phosphorylation did not match those observed for Akt. Early research elucidating the role of Akt as a crucial regulator of cell size depicts a linear Akt-TSC2-mTOR pathway for control of skeletal muscle mass (9, 53). More recently, in addition to the putative role of Akt in derepressing TSC2 inhibition of mTOR activity (10, 54) and Akt's reciprocal regulation with mTOR (47, 59, 60), new evidence also implicates TSC1/2 as an integral mediator of Akt activity (29). Clearly, the intricacy of the regulatory and feed-forward mechanisms within this triumvirate is complex. The present study shows similar exercise-induced changes in $TSC2^{Thr1462}$ and $mTOR^{Ser2448}$ phosphorylation 15 min after exercise independent of the mode of contraction (Fig. 2, *C* and *D*). There was disparity in TSC2 phosphorylation after 3 h of recovery where TSC2 was higher when resistance exercise

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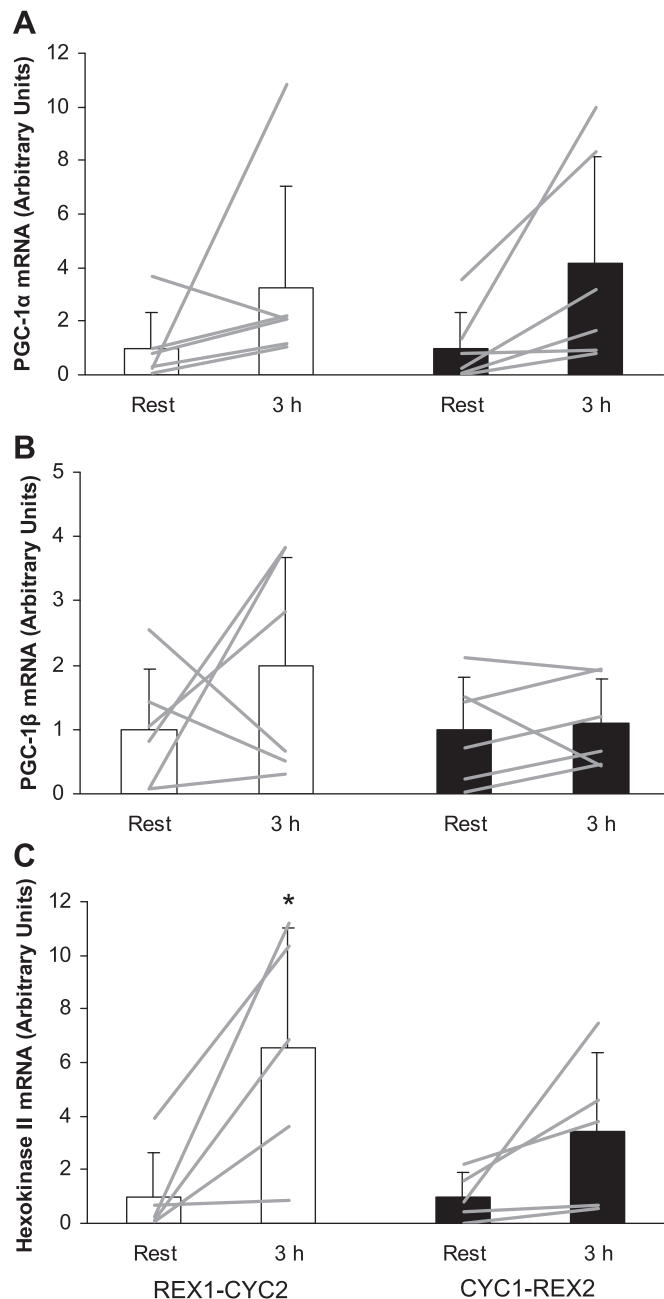

Fig. 5. Peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α; *A*), peroxisome proliferator-activated receptor gamma coactivator-1β (PGC-1β; *B*), and hexokinase II (HKII; *C*) mRNA abundance at rest preexercise and 3 h after cessation of the second exercise bout. Subjects completed 2 experimental trials incorporating consecutive REX (8 × 5 leg extensions at 80% 1 RM) and CYC (30 min at ~70% $\dot{V}O_{2peak}$) bouts performed in alternate order. Results are individual responses and group means (±SD) in arbitrary units. Significant difference ($P < 0.05$) vs. *rest.

preceded cycling (REX1-CYC2; Fig. 2*C*). Given that cycling did not inhibit the Akt response to resistance exercise (Fig. 2, *A* and *B*), the possibility exists that Akt phosphorylation may have been elevated for a longer period when resistance exercise was undertaken before cycling. However, a corresponding exercise order distinction was not apparent for $mTOR^{Ser2448}$ phosphorylation. Indeed, the decrease in mTOR phosphorylation 3 h postexercise was greatest when cycling was subsequent to resistance exercise (REX1-CYC2; Fig. 2*D*). The small number of studies investigating TSC2/mTOR following acute exercise in human skeletal muscle makes comparisons difficult (18, 19, 41, 42). The lack of continuity is likely due to temporal differences in transmitting the signal and subsequent time course of activation. Nonetheless, collectively, our results provide new information regarding a disconnect between Akt phosphorylation and specific target proteins and show comparable changes in TSC2 and mTOR phosphorylation in response to diverse contractile activity.

Translational efficiency of mRNAs with a 5′-terminal oligopyrimidine tract and subsequent protein synthesis has been shown to correlate with p70 S6K and ribosomal protein S6 (rpS6) phosphorylation (56). Although this putative causal relationship remains contentious (56), several studies provide evidence for increased S6K/rpS6 phosphorylation in human skeletal muscle following resistance exercise (14, 18, 19, 32, 33, 42). Intriguingly, we observed increased phosphorylation after an initial exercise bout irrespective of contraction mode and coordinated changes in $S6K^{Thr421/Ser424\text{-}Thr389}$ and rpS6 phosphorylation (Fig. 3). Mascher et al. (41) recently showed an increase in S6K phosphorylation after 60 min of cycling at ~75% maximal O_2 consumption. Taken collectively, these results provide support for the capacity of contractile activity per se rather than merely high-intensity resistance-like stimuli to enhance S6K and rpS6 phosphorylation. Defining causal relationships for the capacity of S6K/rpS6 phosphorylation to induce enhanced translational efficiency and subsequent protein synthesis remain elusive. It has been postulated that reciprocal effects may exist between S6K and rpS6 to promote and repress protein synthesis, respectively, thereby fine-tuning the adaptive signal (56). Furthermore, we have previously shown increased mitogen-activated protein kinase (MAPK) activity after both endurance and resistance exercise (14). MAPK signaling promotes rpS6 phosphorylation at Ser235/6 and stimulates subsequent Cap-dependent translation (55). Thus similar exercise-induced rpS6 phosphorylation likely represents the acute response of the Akt-mTOR-S6K and MAPK pathways converging at Ser235/6. The physiological relevance of the discrepancy between S6K and rpS6 phosphorylation after the second exercise bout is less clear. Phosphorylation of $S6K^{Thr389}$ was sustained when cycling was undertaken after resistance exercise (REX1-CYC2), but this enhanced activity did not correspond with subsequent rpS6 phosphorylation after 3 h of recovery (Fig. 3, *B* and *C*). Regardless, given the capacity of endurance and resistance training to upregulate protein synthesis, it might be expected that contraction per se would stimulate enhanced activity of the translational machinery (64). The results of the present study are consistent with the contraction-induced phosphorylation of S6K-rpS6 and reveal subtle exercise order-dependent changes in the acute phosphorylation responses to combined endurance and resistance exercise.

In addition to the intracellular signaling response, we examined mRNA responses of select genes associated with hypertrophy (5, 66), atrophy (38), and metabolism (49, 50, 52) in skeletal muscle to establish an adaptive profile generated by the consecutive bouts of diverse exercise. A novel finding was that an endurance bout undertaken before resistance exercise attenuated IGF-IEa mRNA (−42%) and also induced small declines in MGF (−27%) and MyoD (−6%) mRNA abundance (Fig.

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4). There is clear evidence for the anabolic effects of IGF (2, 46, 58), and increased IGF-I expression is closely associated with muscle hypertrophy (1). Moreover, increased IGF-IEa and MGF mRNA abundance has been observed after 16 wk of resistance training and corresponded with enhanced mean muscle fiber area (48). In addition, diverse mRNA responses have been reported 24 and 48 h postexercise where IGF-I expression has been shown to decrease and increase after a single bout of resistance exercise (5, 7). Surprisingly, few studies have examined the acute IGF mRNA response to exercise in humans. McKay et al. (45) recently investigated the role of the IGF family members in muscle regeneration after contraction-induced muscle damage. There was no change in MGF/IGF mRNA 4 h after the muscle-damaging protocol, but an increase was observed 24–72 h postcontraction in association with markers of myogenic proliferation and differentiation (45). Psilander et al. (52) and Hameed et al. (23) employed comparable resistance exercise protocols to that used in the present study and determined various IGF-I isoform mRNA abundance after 2 and 2.5 h of recovery, respectively. Psilander et al. observed a decrease in IGF-IEac mRNA, while Hameed et al. saw an increase in IGF-IEc (MGF) mRNA. The findings of the present study represent the collective effect of diverse contraction modes, and it is tempting to speculate that endurance exercise immediately preceding resistance exercise attenuates the anabolic response. However, the paucity of data ensures that the acute IGF mRNA response to endurance and resistance exercise modes remains to be established. MyoD functions as a regulator of the myogenic program within skeletal muscle, and increased MyoD mRNA abundance has been shown in response to resistance and endurance exercise (7, 12, 66). Notably, Yang et al. (66) revealed that both resistance exercise and running induced an approximately eightfold increase in MyoD mRNA 8 h postexercise. Likewise, the cumulative effect of divergent exercise modes on MyoD mRNA abundance in the present study was similar regardless of exercise order (Fig. 4*C*).

Exercise order altered the magnitude of effect on mRNA abundance of genes associated with skeletal muscle inflammation and proteolysis (57). Atrogin and MuRF are ubiquitin ligases that tag specific proteins for proteasomal degradation (8, 57). Our results show elevated atrogin (21%) and MuRF (53%) mRNA when cycling was performed subsequent to resistance exercise (Fig. 4, *C* and *D*). Our group (13) and others (38) have previously shown upregulation of atrogin and MuRF mRNA following a single bout of endurance exercise. Thus our results indicate that endurance activity after resistance training may have the capacity to exacerbate the acute MuRF response and subsequent protein degradation. Conversely, resistance exercise is capable of repressing protein degradation in skeletal muscle, and decreased ubiquitin ligase expression has been observed after a resistance training bout (34, 38, 42). Consequently, when resistance exercise is undertaken after endurance exercise, upregulation of ubiquitin ligase expression may be suppressed. However, the potential pro- and anti-inflammatory effects of these diverse exercise modes with concurrent training are unclear and remain to be established. A potential limitation of the present study was that muscle samples for equivalent 3-h postexercise recovery time points for each individual exercise bout were not taken. Nonetheless, our findings represent an adaptation "snapshot" 3 h after the cessation of contractile activity that characterizes the cumulative effect of the combined divergent stimuli.

The consecutive exercise bouts produced small differences in the mRNA content of genes encoding metabolic proteins, but these minor changes were disparate between PGC-1α, and PGC-1β, and HKII (Fig. 5). Moreover, PGC-1α mRNA abundance was elevated when cycling preceded resistance exercise (~46%, CYC1-REX2), whereas PGC-1β and HKII mRNA were higher when cycling was undertaken after resistance exercise (40–45%, REX1-CYC2). Although PGC-1α/β expression may not be obligatory for enhanced mitochondrial protein content (35), the transient exercise-induced increase in PGC-1 mRNA is closely associated with aerobic adaptation in skeletal muscle (4, 52). Because only minor changes were observed for the metabolically related genes in the present study, the cycling bout used may not have provided sufficient stimulus to elicit mRNA responses typically reported with endurance exercise bouts (49, 51). Therefore, the modest change and differential response in this study provides no clear exercise order-dependent additive or interference effect on metabolic adaptations with concurrent training.

The majority of chronic training studies have shown that concurrent resistance and endurance exercise does not induce optimal specificity of adaptation compared with single-mode training (6, 21, 22, 30, 37). An exception to this evidence is work by McCarthy et al. (43, 44) who utilized similar concurrent training sessions to the present study. Their results show comparable concurrent vs. single-mode resistance and endurance training induced changes in muscle cross-sectional area and strength and $\dot{V}O_{2peak}$, respectively (43, 44). An important distinction between the investigation of McCarthy et al. (44) and the present study was the untrained/sedentary status of the subjects. Indeed, Wilkinson et al. (64) recently showed the capacity of resistance training to enhance mitochondrial adaptation, whereas McCarthy et al. (44) observed modest hypertrophy with endurance training in skeletal muscle of untrained subjects. Therefore, the possibility exists that the cumulative adaptive effect with consecutive resistance and endurance exercise bouts and greater total training volume in previously untrained muscle delays any apparent interference effect with concurrent training. Regardless, more work is needed to fully elucidate the intricacies and time course concerning the specificity of adaptation with concurrent training adaptation.

In summary, this is the first study to examine the acute molecular responses in human skeletal muscle incorporating the cumulative effect of divergent exercise stimuli. Intuitively, both endurance and resistance exercise regulate gene expression and synthesis of proteins and as such contraction per se would stimulate enhanced activity of the translational machinery. Nonetheless, the lack of clear distinction in mTOR-S6K-rpS6 phosphorylation after endurance vs. resistance exercise was unexpected. Indeed, a novel finding of this study was the similar responses in the insulin/IGF pathway signaling with diverse contractile overload. The mRNA data from the present study show modest differential changes in an exercise-order-dependent manner. Of note, our results indicate that endurance activity undertaken before resistance exercise may diminish the anabolic response, whereas performing endurance after resistance exercise may exacerbate inflammation and protein degradation. Although it is difficult to clearly elucidate the specificity of acute responses and subsequent interference within

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the complexity of switching contraction modes and variation in individual responses, the immediacy of diverse bouts of contractile activity in the present study reduced the extent of the desired molecular response compared with individual bouts performed in isolation (14, 18, 23, 50, 52, 66). Indeed, our results provide support for the contention that (acute) concurrent training does not promote optimal activation of pathways to simultaneously promote both anabolic and aerobic responses. Thus undertaking divergent exercise modes in close proximity influences the acute molecular profile and likely exacerbates acute "interference."

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