Reduced carbohydrate availability does not modulate training-induced heat shock protein adaptations but does upregulate oxidative enzyme activity in human skeletal muscle

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Morton JP, Croft L, Bartlett JD, MacLaren DP, Reilly T, Evans L, McArdle A, Drust B. Reduced carbohydrate availability does not modulate training-induced heat shock protein adaptations but does upregulate oxidative enzyme activity in human skeletal muscle. *J Appl Physiol* 106: 1513–1521, 2009. First published March 5, 2009; doi:10.1152/japplphysiol.00003.2009.—The primary aim of the present study was to test the hypothesis that training with reduced carbohydrate availability from both endogenous and exogenous sources provides an enhanced stimulus for training-induced heat shock protein (HSP) adaptations of skeletal muscle. A secondary aim was to investigate the influence of reduced carbohydrate availability on oxidative adaptations and exercise performance. Three groups of recreationally active men performed 6 wk of high-intensity intermittent running occurring four times per week. *Group 1* (*n* = 8; Low + Glu) and *2* (*n* = 7; Low + Pla) trained twice per day, 2 days/wk, and consumed a 6.4% glucose or placebo solution, respectively, immediately before every second training session and at regular intervals throughout exercise. *Group 3* (*n* = 8; Norm) trained once per day, 4 days/wk, and consumed no beverage throughout training. Training induced significant improvements in maximal oxygen uptake ($\dot{V}o_{2\,max}$) (*P* = 0.001) and distance covered on Yo-Yo Intermittent Recovery Test 2 (*P* = 0.001) in all groups, with no difference between conditions. Similarly, training resulted in significant increases in HSP70, HSP60, and αB-crystallin in the gastrocnemius (*P* = 0.03, 0.02, and 0.01, respectively) and vastus lateralis (*P* = 0.01, 0.02, and 0.003, respectively) muscles in all groups, with no difference between conditions. In contrast, training resulted in significant increases in succinate dehydrogenase (SDH) activity of the gastrocnemeius (Low + Glu, Low + Pla, and Norm: 27, 76, and 53% increases, respectively; *P* = 0.001) and vastus lateralis muscles (Low + Glu, Low + Pla, and Norm: 17, 70, and 19% increases, respectively; *P* = 0.001) where the magnitude of increase in SDH activity was significantly larger for both muscles (*P* = 0.03 and 0.04 for gastrocnemius and vastus lateralis, respectively) for subjects training in the Low + Pla condition. Data provide the first evidence that in whole body exercise conditions, carbohydrate availability appears to have no modulating effect on training-induced increases of the HSP content of skeletal muscle. In contrast, training under conditions of reduced carbohydrate availability from both endogenous and exogenous sources provides an enhanced stimulus for inducing oxidative enzyme adaptations of skeletal muscle although this does not translate to improved performance during high-intensity exercise.

stress proteins; glycogen; gastrocnemius; intermittent exercise; peroxisome proliferator-activated receptor-γ coactivator 1-α

HEAT SHOCK PROTEINS (HSPs) are a family of molecular chaperones that are upregulated in the skeletal muscle of both rodents and humans following acute and chronic exercise (24, 25, 30, 33, 36, 40, 42). HSPs can be classified according to their molecular weight, and the prominent HSPs expressed in muscle include the small HSPs (αB-crystallin and HSP27), mitochondrial HSP60, and the highly stress-inducible HSP70. HSP70 is the most conserved of the HSP family (49) and is the most widely studied to date. Given their chaperone properties, HSPs are involved in a number of remodeling processes associated with exercise training, such as facilitating mitochondrial biogenesis (21), regulators of apoptotic pathways (46), and inducing improvements in insulin sensitivity (6). Furthermore, an increased muscle content of HSPs following nondamaging stresses (i.e., preconditioning) provides cytoprotection against subsequent periods of normally lethal or damaging stresses (16, 29, 34, 35). Transgenic approaches have also demonstrated that increased HSP content confers protection against contraction-induced damage in both adult and aged muscle (32). Collectively, such data suggest that increased muscle HSP content evident in trained muscle (42) may be a crucial component of the molecular mechanisms by which regular exercise provides improved cellular function and increased protection against stressful insults.

HSPs were first shown to be expressed following sublethal heat shock (44) although it is now known that stressors, including reduced pH, ischemia, oxidative stress, and depleted energy stores, also increase cellular HSP content (49). Given that these stressors are similar to the homeostatic perturbations occurring in contracting skeletal muscle, it is difficult to determine the precise stressor(s) that is responsible for mediating the exercise-induced production of HSPs. Nevertheless, several authors have proposed that reduced carbohydrate availability from both endogenous (12, 14, 48) and exogenous sources (13) may be a contributing signal, although conclusive evidence is currently limited. Furthermore, no researchers have simultaneously investigated the putative roles of carbohydrate availability from both sources in modulating the exercise-induced stress response. Such research is warranted as it is possible that exercising in conditions of reduced carbohydrate availability may provide an enhanced stimulus for inducing the upregulation of the HSP family of cytoprotective proteins.

One experimental approach to chronically manipulate carbohydrate availability is to have subjects perform exercise training twice daily every second day vs. once daily (17, 54). In this way, all subjects can perform the same amount of work throughout a set training period, yet subjects who train twice every second day commence every second training session with reduced muscle glycogen stores. Interestingly, Hansen

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et al. (17) and Yeo et al. (54) have employed such a model (using one-legged knee extensor and cycling exercise, respectively) and have demonstrated that training in conditions of reduced carbohydrate availability induces significantly greater increases in citrate synthase activity. These data suggest that such training models may have implications for athletic preparation although the latter authors failed to observe increases in exercise performance in elite athletes. Furthermore, these authors have only investigated the effect of reduced muscle glycogen per se and have not simultaneously examined the role of exogenous glucose availability in influencing training adaptations.

The aims of the present study were twofold. Our primary aim was to test the hypothesis that training in conditions of reduced carbohydrate availability from both endogenous and exogenous sources augments the training-induced adaptation of the major HSP families in human skeletal muscle. Given recent studies examining the influence of carbohydrate availability on training-induced athletic performance (17, 54), our secondary aim was to investigate the role of reduced carbohydrate availability on markers of oxidative capacity and exercise performance. We chose a high-intensity intermittent running protocol as our intervention given the relevance of running to the general population (as a keep-fit activity) and also in an array of sporting activities. Furthermore, interval training is receiving increased recognition as an innovative training method compared with traditional methods of continuous running (19, 50).

MATERIALS AND METHODS

Subjects. Thirty recreationally active men volunteered to participate in the study. After giving written informed consent to participate, subjects were randomly assigned (according to Ref. 2) into one of three training groups to provide 10 subjects in each group. Seven subjects withdrew from the study during data collection, and their data were therefore discarded. The characteristics of the remaining subjects who completed the study are shown in Table 1. All subjects refrained from alcohol and caffeine intake for at least 24 h before any of the test sessions (excluding training sessions). None of the subjects had any history of neurological disease or musculoskeletal abnormality, and none was under any pharmacological treatment during the course of the study. The study was approved by the Ethics Committee of Liverpool John Moores University.

Due to subject drop out, there were therefore eight subjects who completed the study in *groups 1* and *3*, and seven subjects who completed the study in *group 2*. Using the NQuery statistical power software (Statistical Solutions, Cork, Ireland) and appropriate statis-

Table 1. *Subject characteristics of each training group*

	Low + Glu (n = 8)	Low + Pla (n = 7)	Norm (n = 8)
Age, yr	20±1	21±1	20±1
Height, m	1.78±0.04	1.75±0.06	1.79±0.03
Body mass, kg	79±7.5	74.5±7.8	77±10.7

Values are means ± SD. Low + Glu and Low + Pla groups trained twice per day, 2 days/wk, and consumed a 6.4% glucose or placebo solution, respectively, immediately before every second training session and at regular intervals throughout exercise and thus performed every second training session with reduced preexercise muscle glycogen levels. Norm group trained once per day, 4 days/wk, consumed no beverage throughout training, and commenced every training session with normal glycogen stores. See *Experimental design* for more details.

Table 2. *Muscle glycogen concentration of the gastrocnemius and vastus lateralis muscles immediately before and after the morning and afternoon training sessions*

	Pre Morning Training	Post Morning Training	Pre Afternoon Training	Post Afternoon Training
Muscle glycogen, mmol/kg wet wt				
Gastrocnemius	96±10	56±8*	54±5	25±10*
Vastus lateralis	90±18	61±15*	59±6	41±13*

Values are means ± SD. Data were compiled from a pilot study (n = 4) and demonstrate that exercise induces significant glycogen depletion in both exercise bouts and that consumption of a low-carbohydrate lunch (consumed at the midpoint of the interval between morning and afternoon exercise) does not promote any glycogen resynthesis before the afternoon session. *Significant difference from respective preexercise values ($P < 0.05$), as assessed by a 1-way repeated measures general linear model.

tical guidelines (3), it was calculated that a sample size of 7 would still enable detection of a mean difference of 50% in basal protein levels assuming an SD of differences equal to 25% and statistical power of 80%. This effect size and SD is based on those values cited in previous studies that have examined training-induced increases in muscle HSP content in both longitudinal (30) or cross-sectional studies (42). Furthermore, with sample sizes of 7, these effect sizes have been abolished when the exercise protocol is performed following nutritional interventions such as reduced carbohydrate availability (14) or antioxidant supplementation (15, 22, 26).

Experimental design. After having initially been assessed for maximal oxygen uptake ($\dot{V}O_{2\ max}$) and running performance on Yo-Yo Intermittent Recovery Test 2 (YoYoIRT2) (see *Assessment of physiological fitness*), subjects completed 6 wk of high-intensity intermittent running occurring four times per week. Each group therefore performed a total of 24 training sessions throughout the 6-wk period. *Groups 1* and *2* trained twice per day, 2 days/wk (once in the morning and once in the afternoon where training sessions were interspersed with a 3- to 4-h rest period), whereas *group 3* trained once per day, 4 days/wk. In this way, each group performed the same amount of work throughout the training period, yet *groups 1* and *2* performed every second training session with reduced preexercise muscle glycogen levels (pilot data demonstrate that subjects commence the afternoon training session with glycogen stores depleted by approximately 35 and 45% in the vastus lateralis and gastrocnemius muscle, respectively; see Table 2). Furthermore, to allow us to also examine the effects of exogenous glucose supplementation on influencing training adaptations, subjects in *group 1* (Low + Glu) consumed a carbohydrate beverage (6.4%) immediately before and throughout every second (i.e., afternoon) training session, whereas subjects in *group 2* (Low + Pla) consumed an identical amount of a taste-, consistency-, and odor-matched placebo solution at identical times. Subjects consumed 8 ml/kg of the relevant solution in a 10- to 15-min period immediately before exercise and a further 3 ml/kg in the active recovery periods following *interval 1* and *interval 3* (see *High-intensity intermittent training protocol* for a description of the training stimulus). In contrast, subjects in *group 3* (Norm) commenced every training session with normal glycogen stores and consumed no beverages during any of their training sessions. Resting muscle biopsies were obtained from the vastus lateralis and gastrocnemius muscles immediately before the first training session (see Fig. 1, B1) and at 72 h after completion of the training program (see Fig. 1, B3). Both vastus lateralis and gastrocnemius muscle samples were analyzed for muscle content of HSP70, HSP60, HSP27, αB-crystallin, Mn superoxide dismutase (MnSOD), cytochrome-*c* oxidase IV (COXIV), peroxisome proliferator-activated receptor-γ coactivator 1-α (PGC1-α), muscle glycogen concentration, and succinate dehydrogenase (SDH)
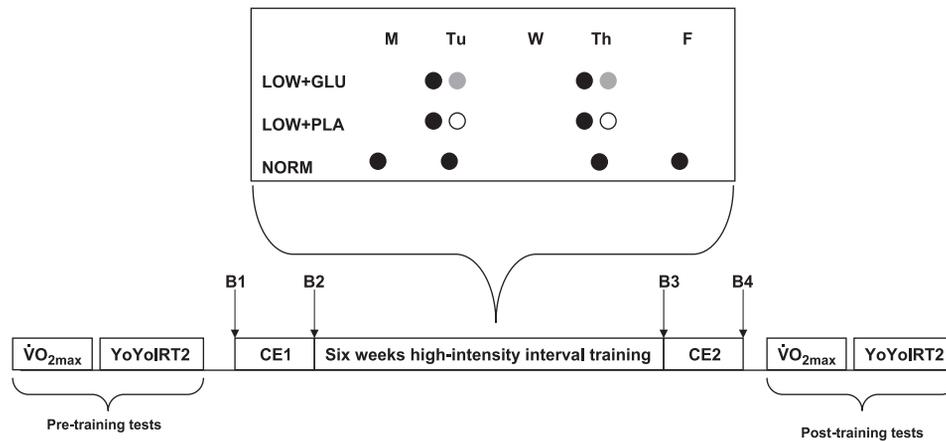
Fig. 1. Schematic illustration of the experimental design. Subjects were initially assessed for maximal oxygen uptake ($\dot{V}O_{2\,max}$) and Yo-Yo Intermittent Recovery Test 2 (YoYoIRT2) performance. Three days after their last pretraining performance test, subjects commenced the training program. The initial training session served as a criterion exercise (CE1) session where muscle biopsies (from the gastrocnemius and vastus lateralis muscles) and venous blood samples were obtained immediately pre- and postexercise (B1 and B2). Subjects trained 4 times/wk, either twice per day on Tuesday (Tu) and Thursday (Th) (Low + Glu and Low + Pla groups) or once daily on Monday (M), Tu, Th, and Friday (F) (Norm group). Three days after the last training session, subjects performed an additional exercise session (CE2) at identical absolute running speeds to the initial training session so as to assess the effects of training on the metabolic cost of a criterion exercise bout. Muscle biopsies and venous blood samples were also obtained immediately pre- and postexercise (B3 and B4). Biopsy samples (from the gastrocnemius and vastus lateralis muscles) obtained at time *points 1* (B1) and *3* (B3) were used to assess the effects of training on resting muscle characteristics. Dark circles, nonsupplemented training session; light gray circles, carbohydrate-supplemented training session; open circles, placebo-supplemented training session.

activity. A schematic illustration of the experimental design is shown in Fig. 1.

Effects of training on the metabolic/physiological cost of a criterion exercise session. To evaluate the effects of training on the physiological and metabolic cost of a criterion exercise session, each subject performed an additional exercise session 3 days after completion of the training program (see Fig. 1, CE2). This session was performed at the same absolute intensities as the first training session (see Fig. 1, CE1). Heart rate was measured continuously during exercise (Polar S610i, Kempele, Finland). Muscle biopsies and venous blood samples were obtained immediately pre- and postexercise for both the first training session (see Fig. 1, B1 and B2) and this additional exercise session (see Fig. 1, B3 and B4). Biopsy samples were assessed for muscle glycogen concentration, and blood samples were analyzed for plasma glucose, lactate, nonesterified fatty acids (NEFA), glycerol, and serum insulin concentration.

Dietary interventions. On each day of testing or training, subjects arrived in the laboratory 2 h after having consumed a standardized breakfast (3.64 MJ, 863 kcal: 63% carbohydrate, 25% fat, 12% protein). For those groups who trained twice per day (i.e., Low + Glu and Low + Pla), the morning and afternoon training sessions were separated by a 3- to 4-h recovery period. At the midpoint of this interval (i.e., 1.5–2 h following completion of the morning exercise session), each subject consumed a standardized low-carbohydrate lunch containing <50 g of carbohydrate (1.29 MJ, 304 kcal: 52% carbohydrate, 39% fat, 9% protein). Pilot data demonstrated that this meal resulted in no significant muscle glycogen resynthesis immediately before the second exercise bout (see Table 2). This dietary intervention therefore allowed the afternoon session to be commenced under conditions of reduced muscle glycogen while simultaneously providing the subjects with some nutritional intake at "lunchtime." Throughout the recovery period, subjects also consumed an adequate amount of water (150% of weight loss) to replace any fluid loss induced by the morning training session (47). To calculate weight loss, subjects were weighed while nude and fully dried immediately before and within 5–10 min after exercise (time scale allowing for biopsy/blood and "drying" procedures) using calibrated precision weighing scales (Seca). Weight loss was measured in kilograms and multiplied by 1.5 to give a value that was subsequently converted to

liters (47). Following the completion of each daily training session (Norm subjects) or every second training session (Low + Glu and Low + Pla), subjects consumed 1.2 g/kg of carbohydrate per hour for 3 h to accelerate muscle glycogen resynthesis before the next exercise bout. These meals were given in the form of sports bars, gels, and drinks (GlaxoSmithKline Consumer Healthcare). After this 3-h period, subjects resumed their habitual diet.

On those days outside of "study" activities, subjects were instructed to consume their habitual dietary intake. To analyze the nutritional intake of such practices, each subject completed three 2-day food diaries at set periods throughout the study period. Subsequent dietary analysis was performed with the software program Microdiet (Downlee Systems). A breakdown of each group's nutritional intake is shown in Table 3. These data demonstrated no significant difference between groups for total energy intake or macronutrient composition (as assessed by a 1-way between-groups general linear model). For 2 days before their pretraining biopsies, subjects consumed their habitual diet. This diet was photocopied, and subjects were instructed to consume an identical diet in the 2 days before their posttraining biopsies. Dietary analysis revealed no differences between groups (as assessed by a 1-way between-groups general linear model), and when taken together, the average composition per subject was ~6.5 ± 0.9 MJ (45 ± 4% carbohydrate, 35 ± 9% fat, 20 ± 7% protein).

To monitor physical activity levels outside of study activities, each subject completed a physical activity diary where they recorded the number of hours of physical activity performed throughout the 6-wk

Table 3. *Nutritional analysis of each group throughout the study period*

	Low + Glu	Low + Pla	Norm
Carbohydrate, %	50±5	53±4	51±3
Fat, %	30±6	27±5	31±5
Protein, %	20±3	20±4	18±4
Energy, MJ	7.5±1.2	8.3±3.2	8.2±2.2

Values are means ± SD. Macronutrient composition is presented as % of total calorie intake. Data were compiled from three 2-day food diaries.

period. There was no significant difference between groups in terms of hours involved in this "additional" physical activity, which equated to approximately 0.5–1 h per subject per week (as assessed by a 1-way between-groups general linear model; data not shown). These activities consisted mainly of walking to and from their place of employment and, occasionally, recreational sporting activities at weekends. Indeed, the majority of subjects reported to the investigators that they did not perform any exercise outside of the study due to the need to recover from the intense nature of the training days.

Assessment of physiological fitness. All subjects were initially assessed for $\dot{V}O_{2 \text{ max}}$ using an incremental exercise test performed on a motorized treadmill (HP Cosmos). Oxygen uptake ($\dot{V}O_2$) was measured continuously during exercise using an on-line gas analysis system (Metamax, Cortex). The test began with a 4-min stage at a treadmill speed of 10 km/h followed by 2-min stages at 12, 14, and 16 km/h. After completion of the 16 km/h stage, the treadmill inclined by 2% every 2 min thereafter until volitional exhaustion despite strong verbal encouragement. The $\dot{V}O_{2 \text{ max}}$ was taken as the highest $\dot{V}O_2$ value obtained in any 10-s period and was stated as being achieved by all of the following end-point criteria: *1*) heart rate within 10 beats/min of age-predicted maximum, *2*) respiratory exchange ratio > 1.1, and *3*) plateau of oxygen consumption despite increasing workload. At least 24 h after completion of this test, subjects were also assessed for intermittent exercise performance using the YoYoIRT2 (27). This test consists of repeated two 20-m runs at a progressively increased running speed controlled by audio bleeps from a tape recorder. Between each running bout, subjects have a 10-s rest period. When the subject twice failed to reach the finishing line in the required time, the distance covered was recorded and represented the test result. The test was performed on an indoor running surface on a 4-m-wide and 20-m-long running lane marked by cones. The coefficient of variation for test-retest reliability of this test in our laboratory is 8.8%. Each subject performed pre- and posttraining assessments of fitness at the same time of day so as to prevent any diurnal variations in performance (45).

High-intensity intermittent training protocol. The intermittent training protocol consisted of high-intensity running and was performed on a motorized treadmill (HP Cosmos). The protocol commenced with a 10-min warm up at a running velocity corresponding to 70% of $\dot{V}O_{2 \text{ max}}$ followed by five 3-min bouts at a running velocity corresponding to 90% of $\dot{V}O_{2 \text{ max}}$. The high-intensity efforts were separated by 3-min active recovery periods (1.5-min at a velocity corresponding to 25% $\dot{V}O_{2 \text{ max}}$ followed by 1.5-min at velocity corresponding to 50% $\dot{V}O_{2 \text{ max}}$). Following the interval and recovery periods, subjects then performed a 10-min cool-down period at a running velocity corresponding to 70% of $\dot{V}O_{2 \text{ max}}$. The exercise protocol therefore gave a total of 15 min of interval exercise and 15 min of active recovery time, thus giving a total time for the intermittent training protocol of 30 min. When including the warm-up and cool-down times, the total duration of the exercise protocol was 50 min. Training intensities were increased by 5% of the original $\dot{V}O_{2 \text{ max}}$ following 2 wk of training and by a further 5% after 4 wk of training. Heart rate was measured continuously during each training session (Polar S610i, Kempele, Finland).

Muscle biopsies and biochemical analysis. Considering that muscles of the lower limb are metabolically more active than those of the thigh during running (9), for comparative purposes we obtained biopsies from both the lateral portion of the gastrocnemius and the midportion of the vastus lateralis muscles. Samples from both muscles were obtained (~30–50 mg wet wt) under local anesthesia (0.5% marcaine) using a Pro-Mag 2.2 biopsy gun (MD-TECH, Manan Medical Products, Northbrook, IL). Once the biopsy needle is inserted through the fascia, the "firing" of the biopsy gun operates with a feed-forward of up to 2.5–3.5 cm, depending on the angle of insertion of the needle. Previous data from our group have demonstrated that the process of serial muscle biopsies per se is insufficient to induce changes in HSP content (25). Moreover, there were ~45 days be-

tween the pretraining and posttraining biopsy (both performed in the same limb and separated by approximately 2–3 cm), therefore further minimizing the impact of repeat biopsies on muscle protein content. Once obtained, samples were immediately frozen in liquid nitrogen and stored at $-80°C$ for later analysis.

For analysis of muscle protein content, samples from both the vastus lateralis and gastrocnemius muscles (~10–15 mg wet wt per muscle) were homogenized separately in a 1% solution of SDS in distilled H_2O containing protease inhibitors (33). Each sample was centrifuged at 4°C and the total protein content of the supernatant was measured using bicinchoninic acid (Sigma Chemical, Dorset, UK). This information was used to standardize the amount of sample loaded onto the gel. Fifty micrograms of total protein was separated by SDS-PAGE using a 12% polyacrylamide gel and 4% stacking gel (National Diagnostics). Proteins were transferred onto a PVDF membrane as previously described (33). The muscle content of HSP70, HSP60, HSP27, αB-crystallin, MnSOD, and PGC1-α was analyzed using a panel of mouse monoclonal (HSP60, HSP70, and COXIV; Sigma, Stressgen and Abcam, respectively) or rabbit polyclonal antibodies (HSP27, αB-crystallin, MnSOD, Stressgen; PGC1-α, Cell Signalling). Samples from each muscle group were run on separate gels and bands were visualized using an enhanced chemiluminescence detection system (Pierce, Rockford, IL, USA) and Chemi-doc image capture system with Quantity One software, where the densities of the entire band were analyzed (Bio-Rad, Hemel Hempsted, UK). Following training, the content of muscle proteins was expressed as a percentage of the pretraining content for each subject.

Muscle glycogen concentration in both the vastus lateralis and gastrocnemius muscles was determined in wet weight (from samples containing ~10–15 mg wet weight per muscle) according to the hexokinase method (31). Muscle SDH activity was assessed in both the vastus lateralis and gastrocnemius muscles (from samples containing ~10–15 mg wet wt per muscle) according to the method described by Kayani et al. (24) where data are presented as micromoles per minute per milligram of protein.

Venous blood samples and biochemical analysis. Blood samples were drawn from a superficial vein in the anticubital crease of the forearm using standard venipuncture techniques (Vacuatiner Systems, Becton, Dickinson). Samples were collected into vacutainers containing EDTA, lithium heparin, or serum separation tubes and either stored on ice or at room temperature (serum samples, ~1 h) until centrifugation at 2,000 rpm for 15 min at 4°C. Following centrifugation, aliquots of plasma/serum were stored at $-80°C$ for later analysis. Samples were analyzed for plasma glucose (Glucose oxidase, Instrumentation Laboratory, Monza, Italy), lactate and glycerol (Randox Laboratories, Antrim, UK), NEFA (NEFA-C, Wako Chemicals, Neuss, Germany), and serum insulin (Insulin ELISA, DRG Instruments) concentration. All postexercise blood samples were corrected for plasma volume changes according to Dill and Costill (11).

Statistical analysis. Statistical analysis was conducted using the Statistical Package for Social Sciences software program (version 15). Changes in $\dot{V}O_{2 \text{ max}}$, YoYoIRT2, and resting muscle characteristics (i.e., stress/mitochondrial protein content, SDH activity, and muscle glycogen concentration) were assessed using a two-way mixed-design repeated-measures general linear model where the within factor was time (pretraining vs. posttraining) and the between factor was training condition (Low + Glu vs. Low + Pla vs. Norm). Changes in the metabolic cost of the criterion exercise session were assessed using a three-way mixed-design repeated-measures general linear model where the within factors were acute exercise response (preexercise vs. postexercise) and training status (pretraining vs. posttraining) and the between factor was training condition (Low + Glu vs. Low + Pla vs. Norm). Where there was a significant interaction effect, Bonferroni post hoc tests were used to locate the differences. All data in text, figures, and tables are presented as means (SD) with P values ≤ 0.05 indicating statistical significance.
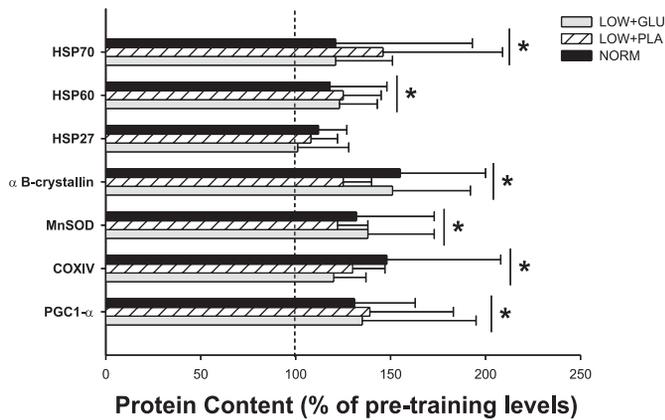
Fig. 2. Posttraining protein content of heat shock protein 70 (HSP70), HSP60, HSP27, αB-crystallin, Mn superoxide dismutase (MnSOD), cytochrome-*c* oxidase IV (COXIV), and peroxisome proliferator-activated receptor-γ coactivator 1-α (PGC1-α) in the gastrocnemius muscle of each group. Data are expressed as a percentage of pretraining values. *Significant main effect of training, *P* < 0.05.
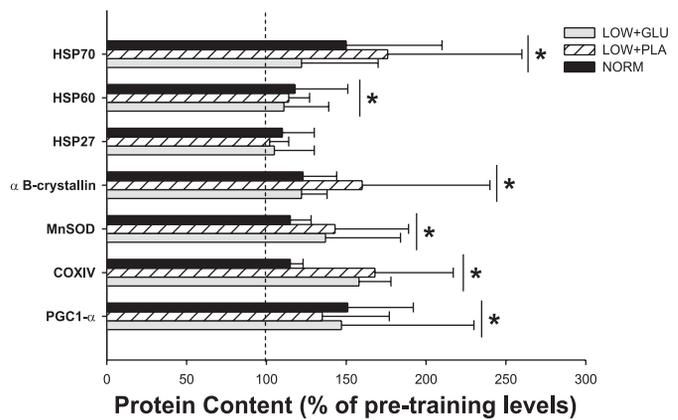

Fig. 3. Posttraining protein content of HSP70, HSP60, HSP27, αB-crystallin, MnSOD, COXIV, and PGC1-α in the vastus lateralis muscle of each group. Data are expressed as a percentage of pretraining values. *Significant main effect of training, *P* < 0.05.

RESULTS

Changes in physiological fitness following training. A comparison of each group's $\dot{V}O_{2\,max}$, time to exhaustion on the $\dot{V}O_{2\,max}$ test, and distance covered on the YoYoIRT2 before and after training are displayed in Table 4. Training improved all performance parameters in all groups ($P = 0.001$ for all indexes) with no difference between training conditions. When data from all subjects were pooled, there was a significant correlation between $\dot{V}O_{2\,max}$ and distance covered on the YoYoIRT2 before and after training ($P = 0.007$ and 0.02, respectively).

Skeletal muscle adaptations following training. The posttraining protein content of HSP70, HSP60, HSP27, αB-crystallin, MnSOD, COXIV, and PGC1-α in the gastrocnemius and vastus lateralis muscle of each training condition is displayed in Figs. 2 and 3, respectively. In all groups, training increased HSP70 ($P = 0.03$), HSP60 ($P = 0.02$), αB-crystallin ($P =$

0.01), MnSOD ($P = 0.001$), COXIV ($P = 0.01$), and PGC1-α content ($P = 0.01$) in the gastrocnemius muscle. Similarly, all of these proteins increased with training in the vastus lateralis muscle (HSP70, $P = 0.01$; HSP60, $P = 0.02$; αB-crystallin, $P = 0.003$; COXIV, $P = 0.001$; MnSOD, $P = 0.001$; PGC1-α, $P = 0.01$). There was, however, no difference between groups in the magnitude of the training-induced increase in the aforementioned proteins in either muscle. In contrast, HSP27 showed no change following training in either muscle.

Training increased SDH activity in both the gastrocnemius ($P = 0.001$) and vastus lateralis ($P = 0.001$) muscles in all groups (see Fig. 4, *A* and *B*, respectively). Specifically, SDH activity increased by 27, 76, and 53% in the gastrocnemius muscle for the Low + Glu, Low + Pla, and Norm conditions, respectively. In the vastus lateralis muscle, SDH activity increased by 17, 70, and 19% in the gastrocnemius muscle for the Low + Glu, Low + Pla, and Norm conditions, respectively. Furthermore, the training-induced increases in SDH activity of the gastrocnemius ($P = 0.03$) and vastus lateralis ($P = 0.04$) were significantly larger for subjects training in Low + Pla compared with the other conditions.

Effects of training on the physiological and metabolic cost of a criterion exercise session. Muscle glycogen utilization of the vastus lateralis and gastrocnemius muscles during the criterion exercise session before and after training is shown in Fig. 5, *A* and *B*, respectively. Training reduced glycogen utilization in the gastrocnemius muscle ($P = 0.04$) during the criterion exercise although there was no difference between groups. In contrast, training did not affect glycogen utilization in the vastus lateralis muscle. Resting muscle glycogen concentration (all data expressed as mmol/kg wet wt) showed no significant change in either the vastus lateralis (pretraining: Low + Glu, 71 ± 18; Low + Pla, 68 ± 21; Norm, 75 ± 14: posttraining: Low + Glu, 79 ± 21; Low + Pla, 74 ± 16; Norm, 78 ± 20) or gastrocnemius muscles following training (pretraining: Low + Glu, 75 ± 12; Low + Pla, 77 ± 17; Norm, 82 ± 14: posttraining: Low + Glu, 81 ± 16; Low + Pla, 73 ± 17; Norm, 77 ± 18).

The responses of the blood metabolites to the criterion exercise session before and after training are shown in Table 5. Acute exercise increased plasma glucose ($P = 0.03$), lactate

Table 4. *$\dot{V}O_{2max}$, run time to exhaustion on $\dot{V}O_{2max}$ test and YoYoIRT2 test performance of each group pre- and posttraining*

	Pretraining	Posttraining	%Increase
$\dot{V}O_{2max}$, ml·kg⁻¹·min⁻¹			
Low + Glu	51.4±4.7	56.5±5.7	10±6*
Low + Pla	56.6±6.7	61.8±4.1	10±8*
Norm	56.9±7.3	60.8±5	8±6*
$\dot{V}O_{2max}$, l/min			
Low + Glu	4.04±0.23	4.4±0.4	9±6*
Low + Pla	4.1±0.57	4.46±0.39	9±6*
Norm	4.23±0.5	4.46±0.52	7±5*
Time to exhaustion, min			
Low + Glu	8.3±1	10.2±0.9	24±11*
Low + Pla	8.7±1.8	10.5±1.4	22±13*
Norm	8.5±1.2	10.9±1.4	24±13*
YoYoIRT2, m			
Low + Glu	400±95	463±113	16±15*
Low + Pla	411±130	474±139	17±10*
Norm	393±132	480±199	20±6*

Values are means ± SD. $\dot{V}O_{2max}$, maximal oxygen uptake; YoYoIRT2, Yo-Yo Intermittent Recovery Test 2. *Significant main effect of training, *P* < 0.05.
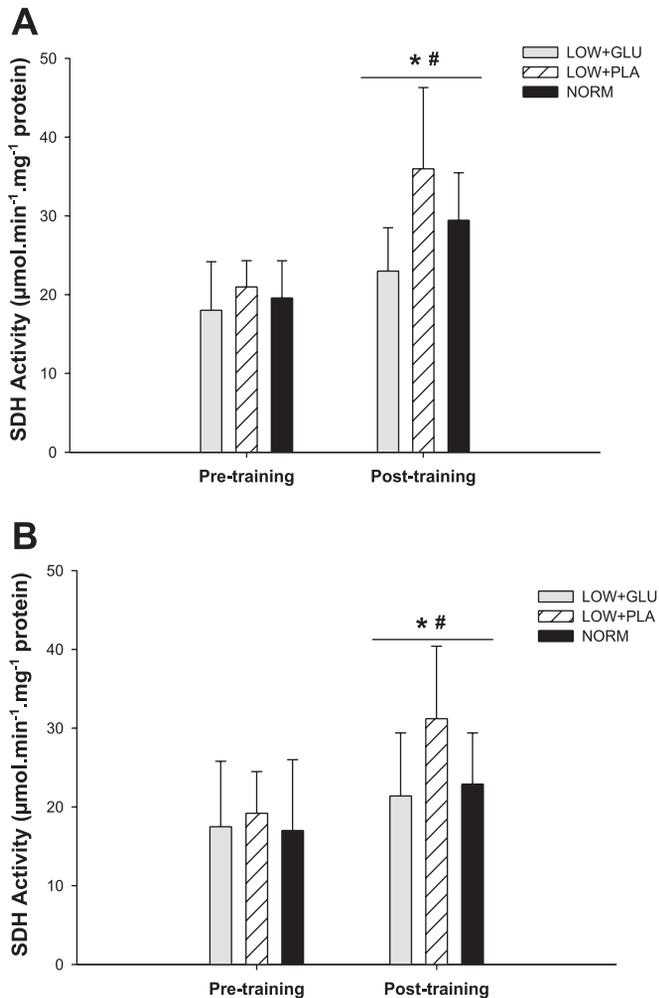
Fig. 4. Succinate dehydrogenase (SDH) activity of the gastrocnemius (*A*) and vastus lateralis (*B*) muscles of each group before and after the training program. *Significant main effect of training, $P < 0.05$. # Significant interaction effect, $P < 0.05$.

muscle. Furthermore, our data confirm and extend the recent findings of Yeo et al. (54) by utilizing a differing training intervention (i.e., high-intensity running vs. cycling) and also demonstrating that reduced muscle glycogen availability augments training-induced increase in oxidative enzyme activity in both vastus lateralis and gastrocnemius muscles. Finally, our data are also novel as we show that consuming exogenous glucose when glycogen stores are low can offset this enhanced training adaptation.

HSPs are a family of highly conserved chaperone-like proteins that protect against contraction-induced damage (32) and are also thought to be involved in facilitating the general remodeling processes inherent to exercise training (38). HSPs are upregulated in response to a multitude of cellular stressors, including hyperthermia, oxidative stress, acidosis, increased intracellular calcium, and energy depletion (49). Given that these stressors are similar to the homeostatic perturbations occurring in contracting skeletal muscle, it is difficult to isolate the precise stressor(s) that is responsible for initiating the exercise-induced induction of HSPs in humans. Using a two-legged knee extensor model in which one limb performed the exercise protocol under conditions of reduced muscle glyco-

$(P = 0.001)$, NEFA $(P = 0.001)$, and glycerol $(P = 0.001)$ and decreased serum insulin (0.002) in both the untrained and trained state. However, only the response of plasma lactate changed with training $(P = 0.001)$ where lactate values were lower following training. There was no difference between groups for any blood metabolite measured.

DISCUSSION

The primary aim of the present study was to test the hypothesis that training in conditions of reduced carbohydrate availability from both endogenous and exogenous sources augments the training-induced adaptation of the major HSP families in human skeletal muscle. Given recent studies examining the influence of carbohydrate availability on training-induced athletic performance (17, 54), our secondary aim was to investigate the role of reduced carbohydrate availability on markers of oxidative capacity and exercise performance. Using an ecologically valid exercise training protocol, our data provide novel findings as we have shown no effect of either endogenous or exogenous carbohydrate availability on modulating training-induced increases in HSP content of skeletal
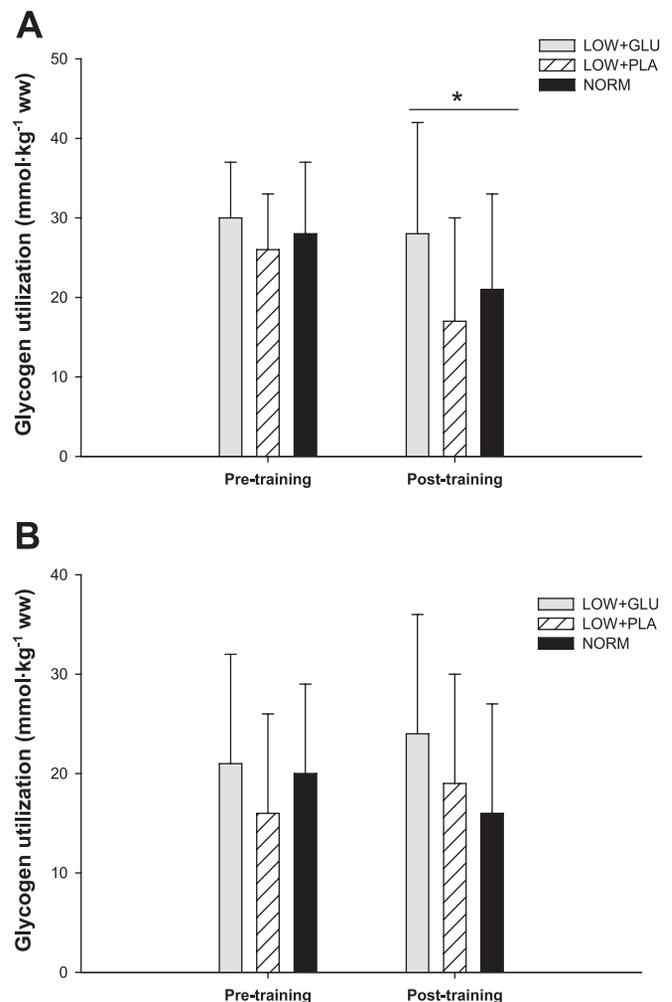

Fig. 5. Muscle glycogen utilization of the gastrocnemius (*A*) and vastus lateralis (*B*) muscles during the criterion exercise session before and after training. ww, wet weight. *Significant main effect of training, $P < 0.05$.

Table 5. *Effects of the training program on the blood glucose, lactate, NEFA, and glycerol (all measured in plasma) and serum insulin response to the criterion exercise session*

	Pretraining		Posttraining	
	Preexercise	Postexercise	Preexercise	Postexercise
Glucose, mmol/l				
Low + Glu	4.6±1.1	5.8±1.4*	4.7±0.8	5.7±0.9*
Low + Pla	4.4±0.7	4.9±1.5*	5.0±0.6	4.6±0.5*
Norm	4.8±0.8	5.3±0.6*	4.6±0.6	4.9±0.5*
Lactate, mmol/l				
Low + Glu	1.7±0.5	5.0±1.8*	1.4±0.3	2.4±1.5*†
Low + Pla	0.9±0.2	2.4±1.1*	0.8±0.3	1.6±1.2*†
Norm	1.2±0.5	3.8±2.3*	1.2±0.5	1.9±1.1*†
NEFA, mmol/l				
Low + Glu	0.15±0.08	0.34±0.13*	0.21±0.09	0.51±0.23*
Low + Pla	0.18±0.11	0.50±0.18*	0.27±0.15	0.51±0.21*
Norm	0.25±0.11	0.58±0.29*	0.21±0.11	0.86±0.53*
Glycerol, μmol/l				
Low + Glu	26±8	111±49*	36±18	139±42*
Low + Pla	31±10	129±19*	49±30	132±62*
Norm	38±22	156±58*	44±21	158±95*
Insulin, μU/ml				
Low + Glu	17.3±14.7	8.3±4.5*	13.6±11.5	7.1±7.4*
Low + Pla	12.7±8.2	4.2±1.3*	14.9±3.3	4.6±2.5*
Norm	12.6±6.0	8.8±3.9*	11.8±7.7	7.1±2.8*

Values are means ± SD. NEFA, nonesterified fatty acids. *Significant main effect of exercise, $P < 0.05$. †Significant main effect of training, $P < 0.05$.

ular HSPs following training is in agreement with us (39) and other authors employing short-term training interventions (30) and also with cross-sectional data from our group (42).

In reviewing the present data in combination with existing literature, it is increasingly apparent that contraction-derived reactive oxygen species (ROS) may be the primary activator of the HSP response during endurance-type exercise protocols as opposed to energy depletion (14) or increases in contracting muscle temperature per se (41). Indeed, a series of studies in humans from our group (22, 25, 26) and others (15) have demonstrated that the exercise-induced upregulation of HSP70 and HSP60 is abolished or attenuated following antioxidant supplementation. In this regard, it is unlikely that carbohydrate availability would have modified the extent of redox signaling between the training conditions employed here. Evidence in support of this view is provided by the finding of a similar training-induced increase in MnSOD content of the gastrocnemius and vastus lateralis muscles between groups. It is suggested that ROS may mediate the exercise-induced induction of HSPs by an indirect effect of oxidation of muscle proteins (33) or a direct effect on heat shock transcription factor 1 (1).

In relation to our second aim, both Hansen et al. (17) and Yeo et al. (54) observed that training under conditions of reduced muscle glycogen availability induces significantly greater increases in citrate synthase activity and resting muscle glycogen concentration of the vastus lateralis muscle (of recreationally active and elite subjects, respectively). The latter authors, however, did not observe improved exercise performance (power output on a 60-min cycle test) as a result of these enhanced metabolic adaptations. The present data confirm and extend these findings as we show (using a high-intensity intermittent running training protocol) that performing 50% of training sessions with reduced muscle glycogen stores induces significantly greater increases in oxidative enzyme activity (as evidenced by SDH activity) of both the vastus lateralis and gastrocnemius muscles, which also does not translate to improvements in high-intensity exercise performance. The enhanced oxidative enzyme activity with no concomitant increase in performance is, however, not surprising given that high-intensity exercise performance is not likely to be limited by enzymes of the tricarboxylic acid cycle. The discrepancy between the previous two studies and no increased resting glycogen concentration in the Low + Pla subjects studied here may be related to subtle differences in methodological design between studies. Before their pretraining biopsy, subjects consumed their habitual diet for 2 days, and this was subsequently photocopied for the subjects to adhere in the 2 days before their posttraining biopsy. Dietary analysis on these diets indicated that the average carbohydrate content per subject was ~45% of 6.5 MJ. The finding of no increased resting glycogen content in Low + Pla subjects may therefore be related to this dietary protocol and the relatively low amount of carbohydrate consumed.

gen, Febbraio et al. (14) observed exercise-induced increases in HSP72 gene and protein expression in the vastus lateralis muscle only in the leg with low glycogen preexercise. The authors therefore suggested that energy depletion may be an important stressor signaling the exercise-induced stress response of human skeletal muscle. Febbraio et al. (13) subsequently investigated the role of exogenous glucose in also influencing HSP muscle adaptation exercise protocol. However, the authors were unable to test this hypothesis as the submaximal cycling exercise protocol that they employed failed to increase HSP72 protein content in the vastus lateralis immediately postexercise (possibly due to insufficient exercise intensity/duration and/or inappropriate sampling time; 40). It is, of course, difficult make comparisons between the aforementioned studies (13, 14) and the present data due to differences in biopsy sampling time point (immediately postexercise vs. 72-h postexercise) and, moreover, duration (acute vs. chronic stress), modality (knee extensor exercise vs. cycling vs. running), intensity (prolonged submaximal contraction/cycling protocols vs. high-intensity intermittent exercise), and the subsequent metabolic responses to the exercise stimulus (18). Indeed, exercise protocols for isolated muscle groups elicit different metabolic responses compared with whole body exercise (18), and, as such, the findings of Febbraio et al. (14) may only be applicable to the contractile protocol investigated.

Nevertheless, the present data demonstrate that in an ecologically valid chronic exercise model, carbohydrate availability from both endogenous and exogenous sources appears to have no modulating effect on the training-induced upregulation of basal heat shock protein content. Rather, we observed comparable training-induced increases between groups in HSP70, HSP60, and αB-crystallin content in both the gastrocnemius and vastus lateralis muscles. The upregulation of these partic-

The increased SDH activity with no increase in COXIV protein is somewhat surprising and may be related to variations in mitochondrial location between samples used for each analysis procedure. Indeed, it is known that SDH activity is twofold greater than COX activity in subsarcolemmal (SS) compared with intermyofibrallar (IMF) mitochondria (8), thus potentially reflecting mitochondria at different stages of biogenesis. Also, SDH activity in the SS region of human vastus lateralis muscle is more responsive to endurance training than IMF region (5).

Our data also provide novel findings as we show that consuming exogenous carbohydrate in the specific low-glycogen training sessions can offset the apparent enhanced training response. This finding agrees with data from acute exercise demonstrating that carbohydrate ingestion during exercise blunts the expression of metabolic genes related to oxidative metabolism (7). Our data are also in agreement with a recent longitudinal training study demonstrating that increased glucose availability during training (achieved via consumption of a high-carbohydrate preexercise meal and consuming glucose during exercise) can attenuate oxidative adaptations of human skeletal muscle (10). Indeed, these authors observed that increased glucose availability before and during each training session completely inhibited the training-induced upregulation of FABPm content (10), a key protein involved in fatty acid metabolism.

To offer some mechanistic insight to the finding of enhanced SDH activity for subjects in Low + Pla, we also measured protein content of PGC1-α before and after training, given its proposed role as a "master" regulator of mitochondrial biogenesis (43). However, in agreement with Yeo et al. (54) our data do not provide definitive support for this pathway as we observed no effect of carbohydrate availability during training on muscle PGC1-α protein content. Furthermore, while Cluberton et al. (7) observed an attenuating effect of glucose ingestion during exercise on oxidative metabolic genes, they also observed no effect of glucose on PGC1-α expression. Collectively, these data demonstrate no modulating effect of carbohydrate from either source on PGC1-α, thus suggesting that the enhanced training response observed by us and others (17, 54) does not appear to be mediated by this pathway. It should be noted, however, that the absolute level of PGC1-α mRNA or protein are not likely to be the main factor determining "activity" as indeed the exercise-induced increase in oxidative enzyme mRNA can occur before an increase in PGC1-α protein (52). Given that both AMP-activated protein kinase (AMPK) (51) and p38 MAPK (4) activity are augmented under conditions of reduced carbohydrate availability and that both proteins are known to increase phosphorylation of PGC1-α (23, 53), one potential mechanism to explain our findings is that increased PGC1-α activity occurred during and/or in the hours following every second training session for subjects training in Low + Pla conditions (hence leading to augmented SDH activity due to activation of these upstream signaling cascades) but yet the actual posttraining levels of PGC1-α protein were similar between groups. Unfortunately, we were unable to test this hypothesis due to the limited tissue provided by our chosen biopsy technique.

Training-induced shifts in substrate utilization are well documented during submaximal exercise intensities corresponding to 60–70% $\dot{V}_{O_2 max}$ (20). In contrast, few authors have examined the changes in substrate metabolism where a high-intensity intermittent exercise bout serves as the criterion exercise session. Mohr et al. (37) observed no differences in postexercise (16 min of speed endurance exercise) glycogen concentration in the vastus lateralis muscle following 8 wk of speed endurance running training, thus suggesting that glycogen utilization was unchanged. The present data appear in agreement with Mohr et al. (37) where glycogen utilization in the vastus lateralis muscle was not significantly reduced following training. In contrast, our data demonstrate a training-induced reduction in glycogen utilization in the gastrocnemius muscle.

The discrepancy between muscles may be related to an enhanced sensitivity of the gastrocnemius muscle to detect local metabolic adaptations to this mode of exercise, likely due to the greater recruitment of the gastrocnemius muscle during running (9). Indeed, consistent with the only other invasive study to compare responses from both muscles (9), we also observed glycogen utilization to be significantly ($P = 0.02$) higher (~30%) in the gastrocnemius compared with the vastus lateralis muscle. Given that posttraining SDH activity was significantly higher for subjects in the Low + Pla condition, one may also expect that the training-induced reduction in muscle glycogen utilization would be more pronounced in these subjects. However, despite the enhanced activity of oxidative enzymes, lipid oxidation cannot be concomitantly increased during high-intensity exercise (28).

In summary, the present data provide the first report that in whole body high-intensity exercise conditions, carbohydrate availability during training has no modulating effect on the training-induced upregulation of the heat shock protein family in human skeletal muscle. In contrast, training with reduced preexercise muscle glycogen levels and without the provision of exogenous carbohydrate provides an enhanced stimulus for inducing beneficial oxidative enzyme adaptations of skeletal muscle.

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