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Effects of dynamic exercise intensity on the activation of hormone-sensitive lipase in human skeletal muscle

Matthew J. Watt, George J. F. Heigenhauser * and Lawrence L. Spriet

*Department of Human Biology and Nutritional Sciences, University of Guelph, Guelph, Ontario N1G 2W1 and * Department of Medicine, McMaster University, Hamilton, Ontario L8N 3Z5, Canada*

It has been proposed that hormone-sensitive lipase (HSL) regulates intramuscular triacylglycerol hydrolysis in skeletal muscle. The primary purpose of this study was to examine the early activation of HSL and the changes in the putative intramuscular and hormonal regulators of HSL activity at various aerobic exercise intensities. Eight male subjects cycled for 10 min at power outputs corresponding to 30, 60 and 90 % peak oxygen uptake ($\dot{V}_{O_2,peak}$). Muscle samples were obtained at rest and following 1 and 10 min of exercise. Intramuscular triacylglycerol (mean ± S.E.M.: 24.3 ± 2.3 mmol (kg dry mass (DM))$^{-1}$), long-chain fatty acyl CoA (13.9 ± 1.4 μmol (kg DM)$^{-1}$) and HSL activity (1.87 ± 0.07 mmol min^{-1} (kg DM)$^{-1}$)) were not different between trials at rest. HSL activity increased at 1 min of exercise at 30 and 60 % $\dot{V}_{O_2,peak}$, and to a greater extent at 90 % $\dot{V}_{O_2,peak}$. HSL activity remained elevated after 10 min of exercise at 30 and 60 % $\dot{V}_{O_2,peak}$, and decreased at 90 % $\dot{V}_{O_2,peak}$ from the rates observed at 1 min (1 min: 3.41 ± 0.3 mmol min^{-1} (kg DM)$^{-1}$; 10 min: 2.92 ± 0.26 mmol min^{-1} (kg DM)$^{-1}$), $P < 0.05$). There were no effects of exercise power output or time on long-chain fatty acyl CoA content. At 90 % $\dot{V}_{O_2,peak}$, skeletal muscle contents of ATP and phosphocreatine were decreased ($P < 0.05$), and free ADP and free AMP were increased ($P < 0.05$) during exercise. No changes in these metabolites occurred at 30 % $\dot{V}_{O_2,peak}$ and only modest changes were observed at 60 % $\dot{V}_{O_2,peak}$. Plasma adrenaline increased ($P < 0.05$) during exercise at 90 % $\dot{V}_{O_2,peak}$ only. These data suggest that a factor related to the onset of exercise (e.g. Ca^{2+}) activates HSL early in exercise. Given the activation of HSL early in exercise, at a time when intramuscular triacylglycerol hydrolysis and fat oxidation are considered to be negligible, we propose that the control of intramuscular triacylglycerol hydrolysis is not solely related to the level of HSL activation, but must also be regulated by postactivational factors.

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Corresponding author M. J. Watt: Department of Human Biology and Nutritional Sciences, University of Guelph, Guelph, Ontario N1G 2W1, Canada, Email: mwatt@uoguelph.ca

Most of the evidence currently available suggests that intramuscular triacylglycerol (IMTG) is available for use as a metabolic substrate, being utilised to the greatest extent during moderate (~60 % peak oxygen uptake, $\dot{V}_{O_2,peak}$) exercise, and at reduced rates at lower (~30 % $\dot{V}_{O_2,peak}$) and higher (~90 % $\dot{V}_{O_2,peak}$) exercise intensities (Hurley *et al.* 1986; Phillips *et al.* 1996; Krssak *et al.* 2000; Decombaz *et al.* 2001; Watt *et al.* 2002*a*). IMTG can be hydrolysed by triacylglycerol lipase or hormone-sensitive lipase (HSL), and monoacylglycerol lipase. HSL is a neutral lipase that cleaves the first and second fatty acids from the glycerol backbone. It is an important control point for IMTG hydrolysis because the activities of HSL and monoacylglycerol lipase for diacylglycerol and monoacylglycerol far exceed the hydrolytic activity of HSL for triacylglycerol (Fredrikson *et al.* 1981; Langfort *et al.* 1999). Despite the detection of HSL mRNA and protein in skeletal muscle almost two decades ago (Holm *et al.* 1987, 1988), the control of HSL and its role in IMTG metabolism remains poorly understood.

An assay for measuring HSL activity in adipose tissue was recently modified and applied to skeletal muscle (Langfort *et al.* 1999). HSL is 'trapped' in its *in vivo* phosphorylation state, and *in vitro* activity is then determined in a stable lipid emulsion. It is important to note that this assay measures the HSL activity *in vitro* and is a measure of the potential catalytic rate (flux) *in vivo*.

Studies conducted using adipose tissue have demonstrated both covalent and allosteric regulation of HSL activity. The putative covalent regulators of HSL that stimulate activation include adrenaline and extracellular signal-regulated kinase (ERK), whereas others such as insulin, 5′-AMP-activated protein kinase (AMPK) and targets of Ca^{2+} such as Ca^{2+}–calmodulin-dependent kinase II (CaMKII) are thought to inhibit HSL activity. Aside from covalent modification, it has been proposed that long-chain fatty acyl CoA (LCFA-CoA) allosterically inhibits (postactivational) HSL activity (for review see Holm *et al.* 2000). In addition, we propose that similar to the control of other rate-limiting enzymes of fuel metabolism, HSL

may be regulated by factors that are related to ATP hydrolysis (e.g. free AMP, free ADP).

In the absence of detailed information pertaining to the measurement and regulation of HSL activity in human skeletal muscle, the primary objective of this study was to examine HSL activity and the changes of the putative intramuscular and hormonal regulators at the onset of exercise of various power outputs. Given the suggested importance of LCFA-CoA for skeletal muscle metabolism (Faergeman & Knudsen, 1997) and in particular IMTG hydrolysis, and given the paucity of data relating to LCFA-CoA accumulation/utilisation during exercise, our secondary aim was to document changes in LCFA-CoA during exercise. In the present study, subjects performed 10 min of exercise at power outputs corresponding to 30, 60 and 90 % $\dot{V}_{O_2,peak}$. These power outputs were also selected to assess the potential effects of Ca^{2+} on HSL activity as the Ca^{2+} signal in skeletal muscle is proportional to the contraction intensity (Westerblad & Allen, 1993). We hypothesised that HSL activity would increase after 1 and 10 min during exercise at 60 % $\dot{V}_{O_2,peak}$, and increase to a lesser extent at 30 and 90 % $\dot{V}_{O_2,peak}$.

METHODS

Subjects

We studied eight healthy males (mean ± S.D. age 25 ± 3 years, body mass 81 ± 5 kg, $\dot{V}_{O_2,peak}$ 48.6 ± 1.2 ml kg^{-1} min^{-1}) who were recreationally active and participated in aerobic exercise 3–5 days week^{-1}. Written informed consent to participate was obtained from each subject after a detailed explanation of the experimental procedures and associated risks were outlined verbally and in writing. The ethics committees of the University of Guelph and McMaster University approved the study, which was performed according to the Declaration of Helsinki.

Pre-experimental protocol

Subjects performed a continuous incremental cycling (Quinton Excalibur, Quinton Instrument, Seattle, USA) test to volitional exhaustion to determine $\dot{V}_{O_2,peak}$. Oxygen uptake ($\dot{V}_{O_2}$) was determined on-line using a metabolic cart (Quinton Q-plex 1, Quinton Instruments, Seattle, USA). Subjects visited the laboratory at least 2 days later and cycled for 10 min at each estimated power output (30, 60 and 90 % $\dot{V}_{O_2,peak}$). $\dot{V}_{O_2}$ was obtained throughout exercise to confirm the power outputs for experimental trials. Dietary records were kept for the 24 h preceding the first experimental trial and subjects were asked to replicate their food intake for the second experimental trial. The average diet composition was 62 ± 2 % carbohydrate, 24 ± 4 % fat and 14 ± 3 % protein. Subjects were instructed to refrain from alcohol, caffeine and exercise for the 24 h preceding each trial.

Experimental protocol

Subjects participated in two experimental trials, each separated by at least 1 week and arrived at the laboratory after an overnight fast (10 h). On one occasion subjects completed 10 min of cycle exercise at either 60 or 90 % $\dot{V}_{O_2,peak}$. On the other occasion, subjects completed 10 min of cycle exercise at 30 % $\dot{V}_{O_2,peak}$, rested quietly for 60 min and then performed a further 10 min of exercise at 60 or 90 % $\dot{V}_{O_2,peak}$. When two exercise bouts were performed on the same day, biopsy samples were obtained from separate legs. Trial days were randomised and counterbalanced.

Upon arrival at the laboratory, a Teflon catheter was inserted into a forearm vein and the catheter was kept patent by flushing with 0.9 % saline. One leg was prepared for percutaneous needle biopsy sampling of the vastus lateralis muscle. Three incisions were made in the skin and deep fascia under local anaesthesia (2 % lidocaine without adrenaline). Immediately prior to exercise, muscle and blood (6 ml) samples were obtained whilst subjects remained on the couch. All muscle samples were rapidly frozen in liquid N_2 for later analysis. When exercise at two different power outputs was performed on the same day, the second leg was prepared for a subsequent needle biopsy procedure to be performed during the 60 min rest between trials.

Subjects commenced cycling at their predetermined power output. Muscle samples were obtained at 1 and 10 min, whilst the subject remained on the cycle ergometer. The time between cessation and recommencing cycling at 1 min was < 30 s. Venous blood samples were obtained at 5 and 10 min of exercise and expired gases were collected on-line between 6 and 9 min for determination of $\dot{V}_{O_2}$ and the respiratory exchange ratio (RER).

Analysis

One portion of heparinised whole blood was centrifuged (2 min, 8000 r.p.m.) and the plasma removed for the determination of insulin by radioimmunoassay (Coat-a-Count insulin test kit, Diagnostics Products, CA, USA). A second portion of blood (1.5 ml) was added to 30 μl of EGTA and reduced glutathione, mixed thoroughly and centrifuged (2 min, 8000 r.p.m.). The supernatant was analysed for plasma adrenaline by radioimmunoassay (Adrenaline RIA, Labor Diagnostika Nord, Germany).

Skeletal muscle was freeze-dried, dissected free of connective tissue, blood and fat under magnification and powdered into one large sample. One aliquot of muscle (6–8 mg) was used for the determination of HSL activity (Langfort *et al.* 1999), with minor modifications. Briefly, the powdered muscle was homogenised on ice in 20 volumes of homogenising buffer using a rotating Teflon pestle on glass. After centrifugation (16 000 *g*, 1 min), the supernatant was removed and stored on ice for immediate analysis of HSL activity. A substrate consisting of 5 mM triolein, 14×10^6 d.p.m. [9, 10-^{3}H]triolein, 0.6 mg phospholipid (phosphatidylcholine–phosphatidylinositol 3:1 w/w), 0.1 M potassium phosphate and 20 % bovine serum albumin was emulsified by sonication (Fredrikson *et al.* 1981; Osterlund *et al.* 1996). The muscle homogenate supernatant (14 μl) was incubated at 37 °C with enzyme dilution buffer (86 μl) and 100 μl of triolein substrate. The reaction was stopped after 20 min by the addition of 3.25 ml of a methanol–chloroform–heptane (10:9:7 v/v/v) solution, and 1.1 ml of 0.1 M potassium carbonate/0.1 M boric acid were added to facilitate the separation of the organic and aqueous phases. The mixture was vortexed, centrifuged at 1100 *g* for 20 min and 1 ml of the upper phase containing the released fatty acids was removed for determination of radioactivity on a beta spectrometer (Beckman LS 5000TA). All measurements were made in triplicate and the mean of these values is reported.

In the present study, HSL activity was normalised to three factors: total muscle protein measured directly from the HSL homogenate supernatant, total muscle creatine obtained directly from the HSL homogenate supernatant and total muscle creatine measured in a perchloric acid (PCA) extract obtained from the same large

aliquot of mixed powdered skeletal muscle. Total protein content was determined using a commercial kit (BCA Protein Assay Reagent Kit, Pierce, Rockford, IL, USA) from an aliquot of the HSL homogenate supernatant, and HSL activity is expressed as nmol min^{-1} (mg protein)$^{-1}$. A second volume of homogenate supernatant was added to 0.5 M PCA to facilitate the degradation of phosphocreatine (PCr). The sample was neutralised with 2.2 M $KHCO_3$ and after centrifugation (15 min, 7000 r.p.m.), a portion of the supernatant was analysed for total creatine by enzymatic spectrophotometric methods (Bergmeyer, 1974).

When expressed as nmol min^{-1} (mg protein)$^{-1}$, the resting and exercise values (60 % $\dot{V}_{O_2,peak}$: rest, 0.84 ± 0.08 nmol min^{-1} (mg protein)$^{-1}$; 1 min, 1.12 ± 0.14 nmol min^{-1} (mg protein)$^{-1}$; 10 min, 1.30 ± 0.08 nmol min^{-1} (mg protein)$^{-1}$) are similar to those reported previously in skeletal muscle from untrained humans during exercise at ~70 % $\dot{V}_{O_2,peak}$ (rest, 0.7 ± 0.2 nmol min^{-1} (mg protein)$^{-1}$; exercise, 1.4 ± 0.3 nmol min^{-1} (mg protein)$^{-1}$; Kjaer *et al.* 2000). In addition, the HSL activity values were not different when normalising to total muscle creatine measured directly from the HSL homogenate supernatant or from the PCA extract ($r^2 = 0.82$, $P < 0.001$, regression not different from $y = x$). We normalised HSL activity to the total creatine content of the muscle homogenate and expressed activity rates as mmol min^{-1} (kg dry mass (DM))$^{-1}$).

A second aliquot of freeze-dried muscle (~10 mg) was extracted in 0.5 M PCA (1 mM EDTA) and neutralised with 2.2 M $KHCO_3$. The supernatant and acid-insoluble pellet were separated and the extract was used for the determination of ATP, PCr, creatine and lactate by spectrophotometric assays (Bergmeyer, 1974; Harris *et al.* 1974).

The acid-insoluble pellet was washed with 1 ml of 0.5 M PCA (1 mM EDTA), centrifuged for 10 min at 2000 *g* and the supernatant was discarded. The pellet was homogenised in 600 μl of 0.2 M KOH containing 10 mM DTT and incubated at 55 °C for 2 h. After cooling the solution on ice, 60 % (w/v) PCA was added and the solution was centrifuged for 10 min at 2000 *g*. The supernatant was removed and neutralised with near-saturated KH_2PO_4 (Alam & Saggerson, 1998). After further centrifugation (10 min at 2000 *g*), the supernatant was removed for radiometric measurement of CoA (Cederblad *et al.* 1990) liberated by the hydrolysis of LCFA-CoA.

A third aliquot of muscle (6 mg) was obtained from all resting samples for the determination of pre-exercise IMTG. Values of IMTG were extracted and the chloroform phase evaporated (Frayn & Maycock, 1980). The IMTG was saponified in ethanolic KOH and the free glycerol was assayed fluorometrically (Bergmeyer, 1974). IMTG was not measured at the conclusion of 10 min of exercise because measurable changes in IMTG content were not expected. All metabolite measurements were normalised to the highest total creatine content from the nine samples obtained for each subject to correct for non-muscle contamination.

Calculations

Free ADP and AMP concentrations were calculated with the assumption of equilibrium of the adenylate kinase and creatine kinase reactions (Dudley *et al.* 1987). Free ADP was calculated using the measured ATP, creatine and PCr values, an estimated H^+ concentration (Sahlin *et al.* 1976) and the creatine kinase equilibrium constant of 1.66×10^9. Free AMP concentration was calculated from the estimated free ADP and measured ATP with the adenylate kinase constant of 1.05.

HSL activation quality control

An *in vitro* assay system was used to measure HSL activity in human skeletal muscle homogenates (Langfort *et al.* 1999). Prior to undertaking our study in human subjects, a series of experiments was conducted to optimise the measurement of HSL activity. A number of lines of evidence suggest strongly that we were measuring HSL and not other neutral lipases. Firstly, pilot studies in which adrenaline was infused into two resting humans (plasma adrenaline ~6 nM) resulted in marked increases in HSL activity that did not increase further when exercise was superimposed (Subject 1: rest, 1.74 mmol min^{-1} (kg DM)$^{-1}$); adrenaline, 3.16 mmol min^{-1} (kg DM)$^{-1}$; adrenaline + exercise, 2.96 mmol min^{-1} (kg DM)$^{-1}$; Subject 2: rest, 2.05 mmol min^{-1} (kg DM)$^{-1}$; adrenaline, 5.11 mmol min^{-1} (kg DM)$^{-1}$; adrenaline + exercise, 5.45 mmol min^{-1} (kg DM)$^{-1}$). This stimulatory response to adrenaline, and the degree of change (i.e. 96 % increase), was almost identical to that observed previously in the isolated rat soleus (Langfort *et al.* 1999) and adrenalectomised humans (Kjaer *et al.* 2000). Secondly, the activation values that we obtained are consistent with those published previously (Langfort *et al.* 1999, 2000; Kjaer *et al.* 2000), which confirms that no significant contamination of adipose tissue HSL was present in our samples.

Time course experiments were conducted to determine the optimal sampling time for the HSL assay. Assays ($n = 4$, data not shown) were performed as described above and stopped at 5 min intervals until 40 min, and thereafter at 60 and 120 min. Our results demonstrated that the HSL reaction slows after 20 min. Therefore, when stopped at 20 min, the results represent the initial maximal rate of the HSL reaction.

Variability of the HSL activity assay was determined in a separate set of experiments. The intra-assay coefficient of variation (CV) for six aliquots, each made in triplicate, determined from one aliquot of freeze-dried muscle was 3.8 ± 1.5 % (mean ± S.E.M.) at 0.95 ± 0.15 nmol min^{-1} (mg protein)$^{-1}$. The interassay CV for an entire data set ($n = 9$) measured on different days from one subject was 10.3 ± 2.6 %.

Homogenisation was performed on separate aliquots ($n = 6$) obtained from the pool of powdered skeletal muscle. Similar values for HSL activity were obtained when using either a polytron homogeniser for 10 s (0.94 ± 0.17 nmol min^{-1} (mg protein)$^{-1}$) or a rotating Teflon pestle on glass for 20 s (0.97 ± 0.20 nmol min^{-1} (mg protein)$^{-1}$).

The effect of substrate availability was also determined on three aliquots from one large aliquot of powdered muscle. Determinations of HSL activity were made when 50, 100 and 200 μl of substrate were made available for HSL. Despite greater substrate availability, HSL activity was similar between experiments (50 μl: 1.32 ± 0.21 nmol min^{-1} (mg protein)$^{-1}$; 100 μl: 1.16 ± 0.13 nmol min^{-1} (mg protein)$^{-1}$; 200 μl: 1.21 ± 0.16 nmol min^{-1} (mg protein)$^{-1}$), indicating that the substrate was not limiting.

In addition to measuring HSL activity against triolein (denoted by TO in previous studies), others have measured HSL activity against a diacylglycerol analogue (referred to as MOME) in an attempt to estimate total enzyme activity (Langfort *et al.* 1999, 2000; Kjaer *et al.* 2000). Since the rate-limiting step in IMTG hydrolysis is the cleaving of the first fatty acid by HSL (Holm *et al.* 2000), the measurement of MOME does not reflect the maximal activity of HSL against the substrate of interest.

Table 1. Levels of muscle metabolites during 10 min exercise at various power outputs

Variable	Trial	0	1	10
ATP (mmol (kg DM)$^{-1}$)	30%	26.1 ± 1.2	27.5 ± 1.5	27.3 ± 1.5
	60%	24.7 ± 0.5	26.4 ± 0.4	26.9 ± 1.2
	90%	27.0 ± 0.9	25.3 ± 1.1*	24.0 ± 1.4*
Phosphocreatine (mmol (kg DM)$^{-1}$)	30%	83.4 ± 2.9	78.8 ± 3.8	80.7 ± 2.5
	60%	75.3 ± 3.9	49.4 ± 3.7*‡	37.7 ± 4.7*‡
	90%	75.0 ± 3.5	47.0 ± 3.7*‡	15.4 ± 3.8*†§
Lactate (mmol (kg DM)$^{-1}$)	30%	6.7 ± 1.3	5.6 ± 1.5	6.5 ± 1.5
	60%	3.9 ± 0.9	24.0 ± 4.7*‡	32.2 ± 6.2‡
	90%	4.6 ± 1.4	45.7 ± 2.0‡§	115.4 ± 8.1*†§
ADP*f* (μmol (kg DM)$^{-1}$)	30%	79 ± 9	112 ± 11	113 ± 9
	60%	94 ± 16	224 ± 26	213 ± 20
	90%	82 ± 5	331 ± 45*‡	698 ± 141*†§
AMP*f* (μmol (kg DM)$^{-1}$)	30%	0.25 ± 0.05	0.41 ± 0.12	0.25 ± 0.03
	60%	0.45 ± 0.07	2.02 ± 0.45	4.32 ± 1.13*‡
	90%	0.47 ± 0.08	1.88 ± 0.44	13.03 ± 3.00*†§
AMP*f*-to-ATP ratio	30%	0.009 ± 0.002	0.016 ± 0.002	0.018 ± 0.002
	60%	0.017 ± 0.005	0.077 ± 0.017	0.079 ± 0.023
	90%	0.010 ± 0.001	0.161 ± 0.041*‡	0.561 ± 0.120*†§

Exercise was carried out at 30, 60 or 90% peak O_2 uptake (denoted 30, 60 and 90%, respectively). Values are means ± S.E.M., $n = 8$. ADP*f*, free ADP; AMP*f*, free AMP. * Significant difference from 0 min of the same trial; † significant difference from 30 and 60% at a given time; ‡ significant difference from 30% at a given time; § significant difference from 1 min of the same trial ($P < 0.05$).

Statistics

Data are expressed as the mean ± S.E.M. Statistical analyses were performed by two-way analysis of variance with repeated measures (time × power output) and specific differences were located using a Student-Newman-Keuls *post hoc* test. Variability is expressed in terms of the CV (where CV = S.D./mean × 100) for determinations of multiple determinations at each time point. The level of statistical significance was set at $P < 0.05$.

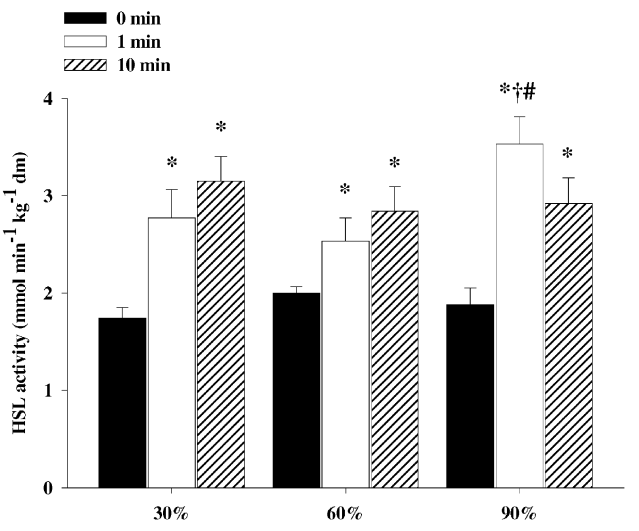

Figure 1. Hormone-sensitive lipase (HSL) activity at rest and during 10 min of exercise at various power outputs

Exercise was performed at 30, 60 and 90 % peak oxygen uptake ($\dot{V}_{O_2,peak}$). Values are presented as means ± S.E.M. ($n = 8$). dm = dry mass. * Significantly different from the resting value of the same trial, $P < 0.05$; † significant difference from 30 and 60 % $\dot{V}_{O_2,peak}$ at the corresponding time point, $P < 0.05$; # significantly different from the value measured at 10 min for the same trial, $P < 0.05$.

RESULTS

Respiratory measures

The $\dot{V}_{O_2}$ at between 6 and 9 min of exercise increased ($P < 0.05$) as a function of power output and averaged 15.2 ± 0.8, 28.9 ± 1.0 and 44.9 ± 1.2 ml kg^{-1} min^{-1}, which equated to 31 ± 1, 59 ± 1 and 92 ± 1 % $\dot{V}_{O_2,peak}$, respectively. The RER averaged 0.87 ± 0.01, 0.94 ± 0.01 and 1.01 ± 0.01 for 30, 60 and 90 % $\dot{V}_{O_2,peak}$, respectively.

HSL activation

HSL activity at rest was not different between trials and averaged 1.87 ± 0.07 mmol min^{-1} (kg DM)$^{-1}$ (Fig. 1). HSL activity increased ($P < 0.05$) from resting values early in exercise (1 min) at all intensities, although the increase during 90 % $\dot{V}_{O_2,peak}$ was greater ($P < 0.05$) than during exercise at 30 and 60 % $\dot{V}_{O_2,peak}$ (Fig. 1). HSL activity at 10 min was not different from 1 min during exercise at 30 and 60 % $\dot{V}_{O_2,peak}$, but decreased ($P < 0.05$) from 1 min during exercise at 90 % $\dot{V}_{O_2,peak}$ (1 min, 3.41 ± 0.32 *vs.* 10 min, 2.92 ± 0.26 mmol min^{-1} (kg DM)$^{-1}$; Fig. 1).

Muscle metabolites

Resting IMTG content was not different between trials and averaged 24.3 ± 2.3 mmol (kg DM)$^{-1}$. LCFA-CoA levels averaged 13.9 ± 1.4 μmol (kg DM)$^{-1}$ at rest across all trials

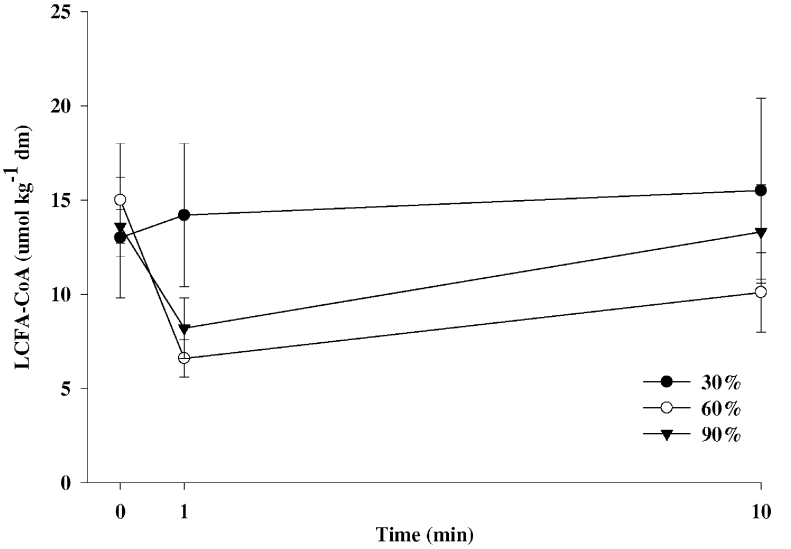

Figure 2. Long-chain fatty acyl CoA (LCFA-CoA) at rest and during 10 min of exercise at various power outputs

Exercise was performed at 30 (●), 60 (○) and 90 % (▼) peak oxygen uptake ($\dot{V}_{O_2,peak}$). Values are presented as means ± S.E.M. ($n = 6$).

and were unchanged by exercise (Fig. 2). Notably, the net reduction in LCFA-CoA at the onset (1 min) of exercise at 60 and 90 % $\dot{V}_{O_2,peak}$ was ~55 % (not significant), whereas no change was evident at 30 % $\dot{V}_{O_2,peak}$.

Muscle ATP content was not affected by exercise at 30 and 60 % $\dot{V}_{O_2,peak}$, whereas ATP levels were decreased ($P < 0.05$) from resting values at 1 and 10 min during 90 % $\dot{V}_{O_2,peak}$ (Table 1). PCr was decreased from resting values at 1 min during exercise at 60 % and 90 % $\dot{V}_{O_2,peak}$ and decreased further during exercise after 10 min at 90 % $\dot{V}_{O_2,peak}$. No changes in PCr were observed during exercise at 30 % $\dot{V}_{O_2,peak}$ (Table 1). Muscle lactate increased after 1 min of exercise at 60 and 90 % $\dot{V}_{O_2,peak}$ and continued to rise during exercise at 90 % $\dot{V}_{O_2,peak}$ such that muscle lactate levels were further elevated ($P < 0.05$) after 10 min (Table 1). Free ADP, free AMP and the AMP-to-ATP ratio were increased from rest at 1 and 10 min during exercise at 90 % $\dot{V}_{O_2,peak}$ (Table 1), whereas no changes were observed at 30 and 60 % $\dot{V}_{O_2,peak}$.

Plasma hormones

Plasma adrenaline was not different from rest during exercise at 30 and 60 % and increased ($P < 0.05$) during exercise at 90 % $\dot{V}_{O_2,peak}$ (Fig. 3). Plasma insulin was not different from rest during exercise at 30 and 60 % $\dot{V}_{O_2,peak}$. In contrast, plasma insulin was decreased ($P < 0.05$) early (5 min) into the exercise bout at 90 % $\dot{V}_{O_2,peak}$ and was further decreased ($P < 0.05$) after 10 min (Fig. 4). The only difference between trials for plasma insulin was observed after 10 min of exercise (30 % > 90 %, $P < 0.05$).

DISCUSSION

The novel findings of the present study were that exercise at 30, 60 and 90 % $\dot{V}_{O_2,peak}$ increased HSL activity at 1 min into the bout, with the increase being greatest at 90 % $\dot{V}_{O_2,peak}$. HSL activity remained constant at 30 and 60 % $\dot{V}_{O_2,peak}$, and decreased slightly after 10 min of exercise at 90 % $\dot{V}_{O_2,peak}$. These findings suggest that human skeletal muscle HSL is initially activated by an intracellular signal that is present at all exercise intensities. Beyond 1 min,

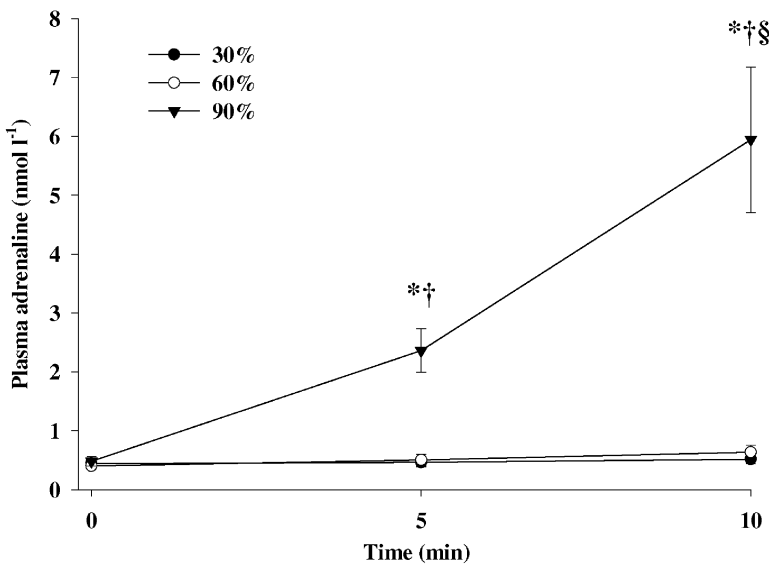

Figure 3. Plasma adrenaline at rest and during 10 min of exercise at various power outputs

Values are presented as means ± S.E.M. ($n = 8$). * Significant difference from 0 min of the same trial; † significant difference from 30 and 60 % $\dot{V}_{O_2,peak}$ at the corresponding time point, $P < 0.05$; § significant difference from 1 min of the same trial ($P < 0.05$).

covalent signals related to ATP hydrolysis (e.g. free AMP) may inhibit further increases in HSL activity, whereas marked changes in β-adrenergic stimulation are unlikely to exert further stimulatory effects. Given the activation of HSL early in exercise, at a time when intramuscular triacylglycerol hydrolysis and fat oxidation are considered to be negligible, we also propose that HSL activation is only one stage of control and other post-activational factors are likely to regulate HSL activity.

Acute regulation of HSL activity during exercise

Studies conducted using adipose tissue suggest that reversible phosphorylation or covalent modification is an important feature of the short-term regulation of HSL activity (Langin *et al.* 1996). Limited information regarding the mechanisms controlling skeletal muscle HSL activation exists partly because *in vitro* studies investigating the control of HSL have not been done. HSL activity increases during maximal tetanic contractions in the isolated rat soleus (Langfort *et al.* 2000). In untrained humans, activation of HSL has been reported to occur during moderate to heavy exercise (73–90 % $\dot{V}_{O_2,peak}$; Kjaer *et al.* 2000). In the same study, HSL activity did not increase during exercise in adrenaline-deficient adrenalectomised patients; however, when adrenaline was infused to mimic normal exercising levels, HSL activity increased (Kjaer *et al.* 2000). These data suggest that adrenaline is important for HSL activation during exercise.

In the present study, HSL activity increased at the onset of exercise at 30 % $\dot{V}_{O_2,peak}$ and remained elevated after 10 min. Aside from the expected increase in cytosolic Ca^{2+}, there were no changes in the measured putative regulators of HSL. At 60 % $\dot{V}_{O_2,peak}$, the increase in HSL activity at 1 min coincided with elevated Ca^{2+}, small decreases in the ATP/free ADP ratio and small increases in free AMP. There were no further changes in HSL activity or the putative regulators after 10 min at 60 % $\dot{V}_{O_2,peak}$. HSL activity during exercise at 90 % $\dot{V}_{O_2,peak}$ was increased at 1 min and was greater than the increases observed at 30 and 60 % $\dot{V}_{O_2,peak}$. The marked increase in HSL activity coincided with increased Ca^{2+} and plasma adrenaline, and large accumulations in the by-products of ATP hydrolysis (e.g. free AMP). HSL activity after 10 min was decreased from 1 min despite further increases in plasma adrenaline.

In the present study we examined three distinct exercise power outputs for two reasons. One was to investigate the *in vivo* effect of varied levels of intracellular Ca^{2+} on HSL activity, and the second was to vary the expectations regarding the use of endogenous fat as a fuel. The finding of an immediate increase of HSL activity at all three of the exercise power outputs examined, and greater elevation of HSL activity during exercise at 90 % $\dot{V}_{O_2,peak}$ was consistent with the premise that Ca^{2+} may be an important early regulator of HSL activity in skeletal muscle. Indeed, Ca^{2+} was the only putative regulator likely to change at all power outputs. Our results are in agreement with a study conducted in the incubated rat soleus that demonstrated a rapid (after 1 min) increase in HSL activity during maximal tetanic contractions (Langfort *et al.* 2000). The magnitude of the increase in the present study (~50 % across intensities) was similar to that observed previously in rat muscle (36 % at 1 min; 50 % at 5 min; Langfort *et al.* 2000). Taken together, these data support an early role for Ca^{2+}-induced activation of HSL in contracting skeletal muscle, probably via stimulation of a protein kinase (e.g. CaMKII). However, elevating Ca^{2+} reduced HSL activity and triacylglycerol hydrolysis in adipocytes (Xue *et al.* 2001), which raises the possibility that another mechanism related to contraction acts to increase HSL activity.

Consistent with the β-adrenergic effect observed on HSL activity and lipolysis in adipose tissue, adrenaline has been proposed to be an important regulator of skeletal muscle HSL activity. A recent study with adrenaline-deficient adrenalectomised patients reported no change in HSL activity during moderate- to high-intensity exercise when

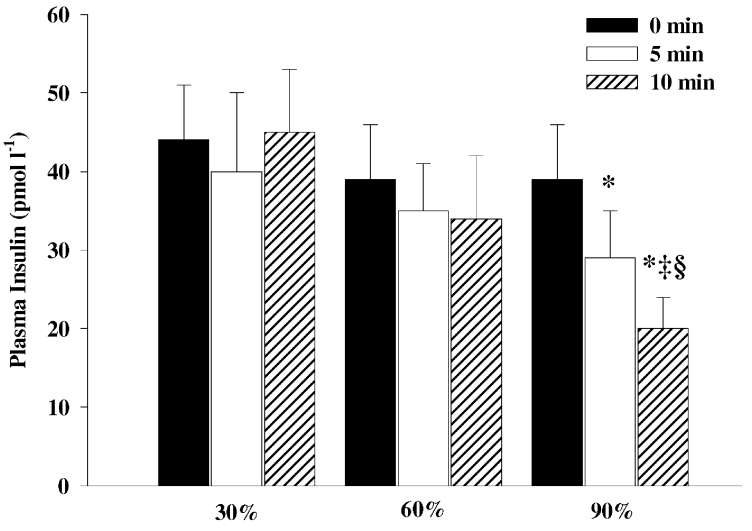

Figure 4. Plasma insulin at rest and during 10 min of exercise at various power outputs

Values are means ± S.E.M. ($n = 8$). * Significant difference from 0 min of the same trial; ‡ significant difference from 30 % $\dot{V}_{O_2,peak}$ at a given time; § significant difference from 1 min of the same trial ($P < 0.05$).

compared with rest. However, when adrenaline was infused to high physiological concentrations (4.5–12.5 nM) the rise in HSL activity mimicked that of the control subjects (Kjaer *et al.* 2000). These data suggest that the metabolic events related to contraction are unable to increase HSL activity and that adrenaline is required for HSL activation in skeletal muscle. Our data do not support this interpretation, as HSL activity was increased from rest during exercise at 30 and 60 % $\dot{V}_{O_2,peak}$ in the absence of changes in plasma adrenaline (Fig. 3). Furthermore, HSL activity was not affected when rat soleus muscles were contracted in the presence of β-adrenergic antagonists (Langfort *et al.* 2000). These data suggest that high concentrations of adrenaline can augment HSL activity, not that adrenaline is required for HSL activation *per se* during exercise.

Plasma insulin is also a powerful inhibitory regulator of adipose HSL activity. The evidence in the present study suggests that although the small decrease in plasma insulin at 90 % $\dot{V}_{O_2,peak}$ may mediate an increase in HSL early in exercise, intramuscular or β-adrenergic effectors are more likely to regulate skeletal muscle HSL activity during the initial minute of exercise.

Aside from covalent modification, which can increase HSL activity, it has been suggested that allosteric regulation (postactivational) of HSL has a major effect on flux through HSL, and subsequently IMTG hydrolysis (Jepson & Yeaman, 1992). Unfortunately, the HSL activation assay does not allow for the observation of allosteric effects that may be occurring *in vivo*, and *in vitro* determination of allosteric effects independent of covalent modification is not currently possible. Given these technical constraints, we sought to measure skeletal muscle LCFA-CoA content during various exercise power outputs and to discuss the potential effects on HSL activity. We assumed that flux through carnitine palmitoyltransferase would be decreased during high-intensity exercise and hypothesised that LCFA-CoA would accumulate at 90 % $\dot{V}_{O_2,peak}$. In the present study we report no change in LCFA-CoA during exercise at all three of the intensities examined and across all time points. These data suggest that LCFA-CoA has no effect on flux through HSL during short-duration exercise.

Accumulations of by-products of ATP hydrolysis during exercise have also been implicated in the activation of metabolic pathways. Skeletal muscle free ADP and AMP increased markedly during exercise at 90 % $\dot{V}_{O_2,peak}$ in the present study, whilst an absence of, or only modest changes were observed at 30 and 60 % $\dot{V}_{O_2,peak}$, respectively. Given that HSL activity increased to a similar degree at all power outputs, a role for covalent regulation by ADP and AMP is unlikely. Free AMP is also an allosteric activator of AMPK (Hardie *et al.* 1999). Binding of AMPK to the so-called basal HSL phosphorylation site in adipose tissue is proposed to inhibit the phosphorylation of active sites and thus inhibit enzyme activity (Garton & Yeaman, 1990). Indeed, the AMPK activator 5-aminoimidazole-4-carboxamide reduced lipolytic activity in adipocytes (Sullivan *et al.* 1994) and endogenous lipid oxidation in the isolated rat soleus (Alam & Saggerson, 1998). Although we have no measure of AMPK, we report no changes in PCr (also an allosteric activator of AMPK), free AMP and HSL activity at between 1 and 10 min of exercise at 30 and 60 % $\dot{V}_{O_2,peak}$. In contrast, at 90 % $\dot{V}_{O_2,peak}$, free AMP was increased and PCr decreased at 1–10 min, suggesting that AMPK exerts an inhibitory effect on HSL activity at this intense power output. In contrast to covalent modification, it is quite possible that free ADP and AMP may also exert powerful allosteric (post-activational) effects given their role in the control of other rate-limiting enzymes. However, this remains to be determined.

HSL activation and IMTG hydrolysis

The bulk of the literature suggests that IMTG is an important metabolic substrate during prolonged low- and moderate-intensity exercise (30–70 % $\dot{V}_{O_2,peak}$), but not at the onset of exercise (after 1 min) or during high-intensity exercise (> 85 % $\dot{V}_{O_2,peak}$; for review see Watt *et al.* 2002*b*). The net change in IMTG was not measured in the present study because conventional techniques cannot determine the expected small changes that occur over a 10 min period (Watt *et al.* 2002*b*). However, the apparent mismatch between early activation of HSL at all power outputs, and IMTG hydrolysis and fat oxidation indicate that factors other than HSL activation are likely to regulate IMTG hydrolysis. Evidence from studies in adipocytes suggest that translocation of HSL from a cytosolic site to the lipid droplet (containing triacylglycerol) is required for lipolysis (Brasaemle *et al.* 2000; Clifford *et al.* 2000). Also in adipose tissue, phosphorylation of perilipins, a family of proteins that coat the surface of lipid droplets, may be critical for access of HSL to triacylglycerol (Souza *et al.* 2002). Thus, HSL activation may not be the only rate-limiting factor regulating IMTG hydrolysis, and various levels of postactivational control (i.e. HSL translocation and perilipin phosphorylation) are likely to be important in mediating IMTG hydrolysis in skeletal muscle.

Conclusion

In summary, human skeletal muscle HSL activation was rapidly increased after 1 min of exercise at 30 and 60 % $\dot{V}_{O_2,peak}$, and to a greater degree at 90 % $\dot{V}_{O_2,peak}$. HSL activity remained elevated at 10 min during exercise at 30 and 60 % $\dot{V}_{O_2,peak}$, but decreased between 1 and 10 min during exercise at 90 % $\dot{V}_{O_2,peak}$. Although the precise regulatory mechanisms cannot be elucidated in the present study, these data suggest that Ca^{2+}-regulated factors (e.g. CaMKII) or other unknown contraction-related factors phosphorylate HSL early in exercise and set the upper limit for flux through the enzyme. Thereafter, signals related to ATP hydrolysis (e.g. free ADP and AMP) may decrease

HSL activity via covalent or allosteric modification, although future studies are required to confirm this. We propose that the control of IMTG hydrolysis is not solely related to the level of HSL activation, but must also be regulated by post-activational factors such as the translocation of HSL to the lipid droplet and phosphorylation of the perilipins.

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