HSP72 gene expression progressively increases in human skeletal muscle during prolonged, exhaustive exercise

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Received 24 March 2000; accepted in final form 28 April 2000

Febbraio, M. A., and I. Koukoulas. HSP72 gene expression progressively increases in human skeletal muscle during prolonged, exhaustive exercise. J Appl Physiol 89: 1055–1060, 2000.—To examine the effect of exercise on heat shock protein (HSP) 72 mRNA expression in skeletal muscle, five healthy humans (20 ± 1 yr; 64 ± 3 kg; peak O2 uptake of 2.55 ± 0.2 l/min) cycled until exhaustion at a workload corresponding to 63% peak O2 uptake. Muscle was sampled from the vastus lateralis, and muscle temperature was measured at rest (R), 10 min of exercise (Min10), ~40 min before fatigue (F-40 = 144 ± 7 min), and fatigue (F = 186 ± 15 min). Muscle samples were analyzed for HSP72 mRNA expression, as well as glycogen and lactate concentration. Muscle temperature increased (P < 0.05) during the first 10 min of exercise but then remained constant for the duration of the exercise. Similarly, lactate concentration increased (P < 0.05) when Min10 was compared with R but decreased (P < 0.05) thereafter, such that concentrations at F-40 and F were not different from those at R. In contrast, muscle glycogen concentration fell progressively throughout exercise (486 ± 74 vs. 25 ± 7 mmol/kg dry weight for R and F, respectively; P < 0.05). HSP72 mRNA was detected at R but did not increase by Min10. However, HSP72 mRNA increased (P < 0.05) 2.2 ± 0.5- and 2.6 ± 0.9-fold, respectively, when F-40 and F were compared with R. These data demonstrate that HSP72 mRNA increases progressively during acute cycling, suggesting that processes that take place throughout concentric exercise are capable of initiating a stress response.

A MAJOR QUESTION IN BIOLOGY is how cells cope with disruption to homeostasis, which, if left unchecked, would lead to irreversible cell damage. To combat protein-related homeostatic disruption, cells respond by synthesizing a family of highly conserved proteins, termed heat shock proteins (HSP), which were first cloned in the 1960s (21). HSP bind to denatured proteins and are involved in the assembly of protein complexes (18), which is why they are also known as “molecular chaperones” (6). HSP are classified into families according to mass (for review, see Ref. 13). The most abundant and widely studied family is the 70-kDa family, which contains the constitutive HSP73 and inducible HSP72 forms.

Some of the conditions known to activate HSP72 include increased temperature (17), ischemia (16), protein degradation (2), hypoxia (7), acidosis (28), reduced glucose availability (24), oxyradical formation (27), and increased intracellular Ca2+. These factors are characteristic of contracting mammalian skeletal muscle cells, and it is, therefore, not surprising that both HSP gene and protein expression are upregulated in the heart (23, 25), liver (23), and skeletal muscle (14, 23, 25) of rodents in response to acute treadmill running. Interestingly, however, no increase in HSP mRNA in rat heart muscles occurs after swimming exercise (8). Whether HSP gene and protein expression increases during concentric exercise in human skeletal muscle is not known. It is possible, therefore, that HSP expression may only increase in human skeletal muscle in response to exercise, which is predominantly eccentric in nature because of the greater susceptibility to protein damage.

Because the increase in temperature and other cellular events take place simultaneously during contraction, it is not clear whether HSP72 expression can increase during exercise independent of changes in contracting cell temperature. Skidmore et al. (25) attempted to address this issue by exercising rats in a cool environment so that colonic temperatures did not differ from the basal state. The HSP72 content of the soleus, extensor digitorum longus, and gastrocnemius muscles was elevated 30 min after exercise, suggesting that factors other than heat that are associated with the exercise can contribute to the accumulation of HSP72. Whereas these data suggest that increased temperature generated during exercise may not be the only factor capable of inducing HSP, it is important to note that an absence of a rise in colonic temperature does not necessarily mean that the temperature of the contracting tissue was stable. Indeed, we have blunted the rise in rectal temperature in humans during exercise in a cool environment and observed a 3.3°C increase in vastus lateralis temperature (5). Further...
research is required to differentiate the effects of elevated temperature and other exercise-induced disruptions to cell homeostasis on HSP gene expression.

Although the effect of exercise on the HSP response in rodents is well documented (8, 12, 14, 15, 23, 25), there are relatively few studies that have examined this phenomenon in humans, and these have produced conflicting results. Acute exercise either increases (22) or has no effect (1) on HSP72 protein expression in human leukocytes. Only one study has previously examined the effect of acute exercise on HSP72 in human skeletal muscle. Puntchart et al. (20) had five humans undertake treadmill exercise for 30 min and examined HSP72 mRNA and total protein content before and after exercise. The authors reported an exercise-induced increase in HSP72 mRNA 4 min after exercise but no increase in total HSP72 protein content. No studies have previously measured HSP gene expression in human skeletal muscle throughout exercise. This is important because cellular events, such as increased temperature, acidosis, and reduced glucose availability, take place throughout exercise. In addition, because protein damage is a major stimulus of HSP expression, it is important to examine HSP gene expression during exercise that is predominantly concentric or nonweight bearing in nature to investigate whether cellular events other than muscle protein degradation or damage can induce a heat shock response.

The purpose of the present study, therefore, was to examine HSP72 mRNA expression in human skeletal muscle progressively throughout steady-state cycling exercise. We also measured some other factors, such as muscle temperature (T_m), glycogen availability, and lactate formation, which are known to stimulate HSP expression in human muscle. We hypothesized that HSP72 gene expression would increase during exercise because factors characteristic of contracting mammalian skeletal muscle cells are known activators of HSP.

MATERIALS AND METHODS

Subjects. Five healthy but untrained humans (20 ± 1 yr; 64 ± 3 kg) volunteered for the experiment. The experimental procedures and possible risks of the study were explained to all subjects before they gave their informed, written consent. The experiment was approved by the Human Research Ethics Committee of The University of Melbourne.

Peak oxygen uptake. Each subject initially performed a cycling test to volitional fatigue on an electromagnetically braked cycle ergometer (Lode Instrument, Groningen, Netherlands) to determine peak oxygen uptake (V\textsubscript{\textcircled{\text{O}}\text{2 peak}}) as previously described (4). V\textsubscript{\textcircled{\text{O}}\text{2 peak}} averaged 2.55 ± 0.2 l/min, and a workload corresponding to ~65% V\textsubscript{\textcircled{\text{O}}\text{2 peak}} was calculated from the oxygen uptake vs. workload regression equation.

Experimental procedure. At least 1 wk after the V\textsubscript{\textcircled{\text{O}}\text{2 peak}} test, subjects returned to the laboratory to perform a familiarization trial. This trial served to familiarize subjects with the cycling protocol and enabled us to check the workload and determine an approximate time to fatigue. Subjects were instructed to refrain from alcohol, caffeine, tobacco, and strenuous exercise and to consume their normal diet for the preceding 24 h. Subjects arrived in the laboratory in the morning after an overnight fast, were weighed, and then commenced cycling on the previously mentioned cycle ergometer at the predetermined workload. The laboratory was maintained at an ambient temperature of 20°C, an electric fan was used to circulate air, and water was provided ad libitum. Subjects were instructed to cycle at the predetermined work rate, maintaining a pedal frequency of 80–90 rpm until fatigue. Fatigue was defined as the inability to complete a full pedal revolution because the work rate on the electrically braked cycle ergometer was pedal-frequency dependent.

The experimental trial was conducted at least 7 days after the familiarization trial. The protocol was identical to the familiarization trial but included muscle sampling at various stages throughout exercise. Muscle samples were obtained from the vastus lateralis by using the percutaneous needle biopsy technique with suction. Briefly, local anesthetic was injected ~10 and 13 cm proximal to the lateral epicondyle of the femur of both legs. Four separate incisions (two in each leg) were then made over the anesthetized areas, and muscle samples were obtained at rest (R), at 10 min of exercise (Min10), ~40 min before fatigue (F-40 min), at fatigue (F = 0 min), and at 144 ± 7 min. F-40 was estimated from the results obtained during the familiarization trial. A sample was obtained at this time to make measurements at a standardized time from the point of fatigue for all subjects. On sampling, the muscle was rapidly frozen in liquid nitrogen for later analysis. At the time of each biopsy, T_m was also determined by inserting a needle thermistor probe (YSI 525, Yellow Springs Instruments, Yellow Springs, OH) 4 cm through the biopsy incision.

Tissue treatment and analysis. Muscle samples were split into two (15- to 50-kg) fragments. One portion was freeze dried for 24 h, dissected free of any blood and connective tissue, powdered, extracted, and analyzed for glycogen and lactate as previously described (4).

Isolation of total RNA. The second muscle fragment was extracted by a modified acid guanidinium thiocyanate-phenol-chloroform extraction method for total RNA (3). Briefly, tissue samples were cut into small (~5 mg) pieces and homogenized for 30 s with 500 μl of RNAzol B (Tel-test, Friendswood, TX) in round-bottom sterile tubes. Fifty microliters of chloroform/soamylalcohol (24:1) were subsequently added to the homogenates, mixed for 15 s, and left on ice for 5 min. The samples were subsequently spun at 18,000 g at 4°C for 15 min. The upper aqueous phase containing the total RNA was transferred to a sterile tube, and, after the addition of an equal volume of ice-cold isopropanol, the samples were placed at −20°C for 60 min. After this, 1 μl of 20 mg/ml glycogen was added for the aid of pelleting the total RNA and the solution mixed before centrifugation at 18,000 g at 4°C for 15 min. The supernatant was aspirated off, and 500 μl of 75% ethanol were added to rinse the samples, which were subsequently spun at 10,000 g at 4°C for 10 min. The supernatant was removed, and the total RNA pellets were air dried before being resuspended in water (0.05% diethyl pyrocarbonate treated). The total RNA content and purity were established by measuring absorbance readings at 260 and 280 nm.

To visualize the integrity of the total RNA, 0.5 μg was fractionated on a 1% denaturing agarose gel. The total RNA was subsequently quantified two to three more times before it was DNase treated to remove residual genomic DNA contamination by using a DNase I amplification grade kit (Life Technologies, Gaithersburg, MD).

RT-PCR. For each total RNA sample, 0.1 μg was reverse transcribed in a 10-μl reaction containing 1 × TaqMan RT buffer, 5.5 mM MgCl\textsubscript{2}, 500 μM each 2′-deoxynucleoside 5′-triphosphate, 2.5 μM random hexamers, 0.4 U/μl RNase A, 0.4 U/μl RNase T\textsubscript{1}, and 100 ng of 0.2 U/μl MultiScribe reverse transcriptase (Applied Biosystems, Foster City, CA). The reaction mixture was placed at 4°C for 10 min, and at 95°C for 15 min. The sample was subsequently adjusted to a final volume of 20 μl containing 12.5 μl of 2× TaqMan universal PCR master mix (Applied Biosystems) and 100 ng of 0.2 U/μl TaqMan universal PCR master mix (Applied Biosystems). The sample was placed at 4°C for 5 min, and at 95°C for 15 min. The sample was subsequently adjusted to a final volume of 20 μl containing 12.5 μl of 2× TaqMan universal PCR master mix (Applied Biosystems) and 100 ng of 0.2 U/μl TaqMan universal PCR master mix (Applied Biosystems). The sample was placed at 4°C for 5 min, and at 95°C for 15 min. The sample was subsequently adjusted to a final volume of 20 μl containing 12.5 μl of 2× TaqMan universal PCR master mix (Applied Biosystems) and 100 ng of 0.2 U/μl TaqMan universal PCR master mix (Applied Biosystems). The sample was placed at 4°C for 5 min, and at 95°C for 15 min. The sample was subsequently adjusted to a final volume of 20 μl containing 12.5 μl of 2× TaqMan universal PCR master mix (Applied Biosystems) and 100 ng of 0.2 U/μl TaqMan universal PCR master mix (Applied Biosystems).

This study was supported by the Australian Government Department of Health and Ageing, the Victorian Government Department of Health and Human Services, the National Health and Medical Research Council of Australia (grant 671149 to L. V. Cherry), and the Blatchford Foundation.
inhibitor, and 1.25 MultiScribe RT (PE Biosystems, Foster City, CA). Although samples were DNase treated, control samples were analyzed when total RNA samples received all reagents except the RT. The reverse transcription reactions were performed by using a GeneAmp PCR system 9600 (PE Biosystems) with conditions at 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min. Each reaction was then diluted 1:10 in 0.01 M EDTA, pH 8.0, and stored at −80°C until further use.

Rather than utilize conventional PCR approaches to study gene expression, we were able to quantitate HSP72 mRNA by real-time PCR using an ABI PRISM 7700 sequence detector (PE Biosystems). This technique, pioneered in the early 1990s (10), allows for the characterization of mRNA by the cycle number at which PCR product accumulation is first detected by fluorescence during cycling, rather than the amount of PCR product accumulated after a fixed number of cycles (9). Therefore, real-time PCR generates quantitative data because PCR fidelity is highest at early cycles. The elimination of post-PCR procedures reduces the risk of contamination and errors, which ultimately leads to highly reproducible and quantitative results.

In this study, a multiplex comparative critical threshold (Ct) method was employed, in which a Ct value reflects the cycle number at which the DNA amplification is first detected. In each multiplex reaction, both human HSP72 and 18S were limited for 18S. This was possible because of the different detection by fluorescence during cycling, rather than the amount of PCR product accumulated after a fixed number of cycles (9). Therefore, real-time PCR generates quantitative data because PCR fidelity is highest at early cycles. The elimination of post-PCR procedures reduces the risk of contamination and errors, which ultimately leads to highly reproducible and quantitative results.

To perform real-time PCR, human HSP72 probe and primers were designed by using Primer Express version 1.0 (PE Biosystems). A 72-bp fragment was amplified by using the forward primer 5′-ACCAGAGACAGGAGATCTTC-3′ and the reverse primer 5′-GCCCTCGTACCTGATC-3′ (Sigma Genosys, Castle Hill, NSW, Australia). A TaqMan fluorescent probe, 5′-FAM (6-carboxy fluorescein)-CCTACTCGAAAC-CAACCCTGG-3′ TAMRA (6-carboxy-tetramethylrhodamine) (PE Biosystems), was included with the primers in each reaction. The TaqMan probe and primers for 18S were supplied in a control reagent kit (PE Biosystems).

PCR reactions were carried out in 25-μl volumes consisting of 1× TaqMan universal PCR master mix (including passive reference), 50 nM TaqMan 18S probe, 20 nM 18S forward primer, 80 nM 18S reverse primer, 100 nM TaqMan human HSP72 probe, and 900 nM human HSP72 forward and reverse primers. The concentrations of the HSP72 probe and primers were chosen based on pilot analyses in which optimal concentrations were determined. cDNA (5 ng) and preparations without RT were amplified by using the following conditions: 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Comparative Ct calculations for the expression of HSP72 were all relative to the resting sample for each subject. For each sample, 18S Ct values were subtracted from HSP72 Ct values to derive a ΔCt value. The resting value for each subject was then subtracted from the exercise samples for each subject to derive a ΔΔCt value. The expression of human HSP72 relative to the resting sample was then evaluated by using the expression 2−ΔΔCt. The coefficient of variation, determined on the Ct value of the 18S samples for each of the four samples for each subject, was <1%. In addition, our laboratory has previously demonstrated that the intra-assay coefficient of variation for one target gene is <5% (11).

Statistical analyses. Data were examined for normality of distribution by using a normal probability plot. The analyses revealed that Tm, muscle glycogen, and lactate were normally distributed. However, data for HSP72 mRNA were not normally distributed. To ensure data homogeneity for this variable, data were log transformed before statistical analyses. A one-way ANOVA with repeated measures on the time factor was used to compare the data throughout the trial. A Newman-Keuls post hoc test was used to locate difference when the ANOVA revealed a significant interaction. All data are reported as means ± SE unless otherwise stated. The level of significance for all tests was set at P < 0.05.

RESULTS

Subjects cycled for 186 ± 15 min at a workload that corresponded to 63% V̇O2peak. Tm increased (P < 0.05) during the first 10 min of exercise. However, subsequent to this time, there was no further increase in Tm (Fig. 1).

Similarly, muscle lactate increased (P < 0.05) when Min10 is compared with R. It subsequently fell such that the concentrations at F-40 and F were not different when compared with R (Fig. 2). In contrast, muscle glycogen concentration decreased (P < 0.05) progressively throughout exercise, and concentrations were very low (<50 mmol glucosyl U/kg dry wt) at F (Fig. 3). HSP72 mRNA was detected in all resting samples; however, within 10 min of exercise, there was no increase in gene expression. HSP72 mRNA increased 2.2-fold (P < 0.05) and 2.6-fold (P < 0.05), respectively, when F-40 and F are compared with R (Fig. 4).

[Graph showing muscle temperature during cycling exercise to fatigue at 63% V̇O2peak] Values are means ± SE (n = 5). *Significant difference (P < 0.05) compared with rest.

Fig. 1. Muscle temperature during cycling exercise to fatigue at 63% peak O2 uptake (V̇O2peak). Values are means ± SE (n = 5). *Significant difference (P < 0.05) compared with rest.
DISCUSSION

This study is the first to demonstrate that HSP72 mRNA increases progressively in human skeletal muscle during prolonged exercise. Despite a marked increase in Tm and lactate accumulation in the first 10 min of exercise, HSP72 mRNA did not increase. However, even though Tm did not increase and intramuscular lactate content fell to resting levels beyond 10 min of exercise, HSP72 mRNA increased after this time (Fig. 4). Interestingly, this increase coincided with reduced intramuscular glycogen availability.

Only one previous study has attempted to quantify HSP72 in human muscle in response to an acute exercise bout. Using fixed cycle PCR methodology, Puntschart et al. (20) measured HSP72 mRNA and protein expression in five men before and after a 30-min run. These authors reported a fourfold increase in HSP72 mRNA 4 min after exercise. This increase was exacerbated after 30 min of recovery and was still elevated threefold 3 h after exercise. Interestingly, the increase in gene transcription did not result in any protein translation 3 h postexercise. In the present study, we did not measure HSP72 protein because of insufficient tissue. Although it would have been desirable to measure protein levels, it was unlikely, based on the previous study (20), that we would have observed any increase in protein expression during the exercise bout. Further work that samples muscle into recovery for periods longer than 3 h is necessary to determine the time course for HSP gene transcription to protein translation in human muscle in response to a single bout of acute exercise.

To our knowledge, this is the first study to use real-time PCR for the study of gene expression in human skeletal muscle in response to exercise. We established that as little as 0.05 ng of starting material per PCR reaction could be used for quantitation of the human HSP72 gene. This is a result of the fluorescence being detected by the sequence detector system very early on during cycling at the beginning of the exponential phase of PCR, when PCR fidelity is highest. Hence, this technique allows for the precise quantitation of many genes from a small (<20-mg) percutaneous muscle biopsy. It has, therefore, good potential for the study of human muscle gene expression not only in response to exercise but also in pathogenesis.

Although, in the present study, we used a more precise method for measuring gene expression than did Puntschart et al. (20), it is interesting to compare the previous data with ours. The magnitude of the increase in our study was less than threefold (Fig. 4), compared with an ~4.5-fold increase reported previously (20). It is important to note that a major difference when the two studies are compared was the mode of exercise chosen. In the previous study, subjects ran on a treadmill, whereas our subjects cycled on an ergometer in the present study. It is well known that running entails an eccentric component to contraction, whereas
cycling primarily involves concentric contractions. This appears to be an important difference with respect to the disruption to cellular homeostasis. Indeed, after 2 h of running exercise, plasma interleukin-6, a cytokine released at the site of inflammation, increases to ~100 pg/ml (19). In contrast, we have recently observed that a similar duration and intensity of cycling exercise results in a plasma interleukin-6 concentration of <2 pg/ml (26). Hence, the differences observed when HSP72 mRNA expression in the present study is compared with the results of Puntschart et al. (20) may be related to the mode of exercise and the amount of protein damage associated with the exercise. Notwithstanding the fact that the increase in HSP72 mRNA was lower in the present study, compared with the previous study (20), it was nonetheless elevated. This demonstrates that changes that occur within muscle cells during exercise that result in minimal damage are capable of initiating a heat shock response.

It has previously been demonstrated that the HSP72 content of the contracting rodent muscle was elevated 30 min after exercise in the absence of any exercise-induced increase in colonic temperature. The authors suggested that factors other than heat that are associated with the exercise may contribute to the accumulation of HSP72 (25), although the absence of a rise in colonic temperature does not necessarily mean that the temperature of the contracting tissue was stable (5). In the present study, Tm did not increase beyond 10 min, but HSP72 mRNA markedly increased thereafter. Given these results, it is tempting to speculate that, during exercise, cellular events other than an increase in cell temperature can induce a “heat shock” response. However, our data cannot determine whether the increase in HSP72 mRNA was due to factors other than cell temperature, because Tm increased in the first 10 min of exercise. It is possible that a time lag existed between the stressor (elevated Tm) and gene transcription. Hence, we cannot dismiss the early increase in Tm being responsible for the augmented gene expression late in exercise. To conclusively test the hypothesis that cellular events other than an increase in contracting cell temperature can induce a heat shock response, the Tm must be clamped at basal levels throughout exercise. This is technically very difficult to achieve because contracting muscle is the major site for heat production during exercise.

Of note, the increase in HSP72 gene expression occurred late in exercise when intramuscular glycogenolysis was reduced to low levels (Fig. 3). It has been demonstrated previously that reduced glucose availability is capable of inducing a heat shock response (24), and our data are consistent with the hypothesis that HSP gene expression in contracting muscle during exercise increases when glucose availability is compromised.

In summary, we have observed that HSP72 gene expression progressively increases during submaximal exercise, suggesting that cellular processes that take place throughout prolonged concentric exercise are capable of initiating a heat shock response.

We acknowledge the medical assistance of Dr. Andrew Garnham and the technical assistance of Jane Danczy in collecting the tissue samples. We also acknowledge the assistance of Robin Murphy for advice in refining the RNA extraction procedure.

The following foundations contributed to the purchase of the ABI PRISM 7700 sequence detector: The Sylvia and Charles Viertel Foundation, The Clive and Vera Ramaciotti Foundation, The Philip Bushell Foundation, and The Harold and Cora Brennen Benevolent Trust. We thank them.

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