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Impact of Hot and Cold Exposure on Human Skeletal Muscle Gene Expression

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Abstract

Many human diseases lead to a loss of skeletal muscle metabolic function and mass. Local and environmental temperature can modulate the exercise-stimulated response of several genes involved in mitochondrial biogenesis and skeletal muscle function in a human model. However, the impact of environmental temperature, independent of exercise, has not been addressed in a human model. Thus, the purpose of this study was to compare the effects of exposure to hot, cold, and room temperature conditions on skeletal muscle gene expression related to mitochondrial biogenesis and muscle mass.

METHODS—Recreationally trained male subjects ($n=12$) had muscle biopsies taken from the *vastus lateralis* before and after 3 h exposure to hot ($33\text{ }^{\circ}\text{C}$), cold ($7\text{ }^{\circ}\text{C}$), or room temperature ($20\text{ }^{\circ}\text{C}$) conditions.

RESULTS—Temperature had no effect on most of the genes related to mitochondrial biogenesis, myogenesis, or proteolysis ($p > 0.05$). Core temperature was significantly higher in hot and cold environments compared to room temperature ($37.2 \pm 0.1\text{ }^{\circ}\text{C}$, $p = 0.001$; $37.1 \pm 0.1\text{ }^{\circ}\text{C}$, $p = 0.013$; $36.9 \pm 0.1\text{ }^{\circ}\text{C}$, respectively). Whole body oxygen consumption was also significantly higher in hot and cold compared to room temperature ($0.38 \pm 0.01\text{ L}\cdot\text{min}^{-1}$, $p < 0.001$; $0.52 \pm 0.03\text{ L}\cdot\text{min}^{-1}$, $p < 0.001$; $0.35 \pm 0.01\text{ L}\cdot\text{min}^{-1}$, respectively).

CONCLUSIONS—These data show that acute temperature exposure alone does not elicit significant changes in skeletal muscle gene expression. When considered in conjunction with previous research, exercise appears to be a necessary component to observe gene expression alterations between different environmental temperatures in humans.

Keywords

Temperature; mitochondrial biogenesis; myogenesis; proteolysis; mRNA

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors report no potential conflicts of interest associated with this manuscript.

INTRODUCTION

Mitochondrial dysfunction has widespread deleterious effects on cellular function and plays a critical role in the pathologies of many diseases, including aging (Ames et al. 1993), peripheral arterial disease (Pedersen et al. 2009), and diabetes (Fosslien 2001; Kelley et al. 2002; West 2000). These chronic diseases are also associated with loss of skeletal muscle function and mass that can subsequently contribute to a variety of additional health concerns, such as sarcopenia and insulin resistance (Lee et al. 1998). The maintenance of skeletal muscle function is critical for functional independence and quality of life. Muscle atrophy ultimately leads to limited mobility, increased risk of injury from falling, slow gait, and very poor physical endurance (Burton and Sumukadas 2010). Understanding the mechanisms contributing to mitochondrial pathologies and subsequent loss of skeletal muscle function is clinically important, particularly due to the overall aging of the population and the increasing number of individuals suffering from chronic disease.

Based on the known mechanisms underlying maintenance of mitochondrial function and skeletal muscle mass, exercise has been successfully implemented in clinical populations to partially ameliorate the deleterious effects of disease conditions (Kosek et al. 2006). Although exercise alone is a valuable therapy to stimulate mitochondrial development and myogenic pathways, reduced capacity to perform adequate exercise in a sedentary or diseased population illustrates the need for further development of novel therapies to stimulate mitochondrial development and muscle growth.

Environmental temperature may play an important role in regulating mitochondrial biogenesis. Peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1 α), a transcriptional co-activator known as a master regulator of metabolism and mitochondrial biogenesis, has previously been shown to be sensitive to temperature. Specifically, exercise and subsequent recovery in a cold environment leads to enhanced expression of PGC1- α and alterations in other key genes associated with mitochondrial development (Slivka et al. 2012). In addition, our preliminary data indicate that local cold application during and following resistance exercise may inhibit myogenic gene expression and increases the expression of genes related to proteolysis (Shute et al. 2015). Furthermore, local heat application decreases forkhead box O3 (FOXO3) mRNA, suggesting a reduction in proteolytic activity, when compared to the cold application (Shute et al. 2015).

Temperature manipulation alone could be a viable alternative to exercise, however studies in rodents found that metabolic changes with cold exposure are critically dependent upon the presence of an exercise stimulus (Goto et al. 2000; Seebacher and Glanville 2010). Determining the optimal stimuli and acute outcomes associated with temperature exposure in humans will point the way toward the development of effective and novel protocols for the treatment of pathologies related to mitochondrial dysfunction and skeletal muscle disorders. Furthermore, preliminary results indicate that local heating of skeletal muscle may enhance the myogenic response to acute resistance exercise over that of local cooling (Shute et al. 2015). However, no data are currently available to indicate if this temperature-related response can be manifested with environmental temperature alterations without an exercise stimulus in humans. Therefore, the purpose of this study was to determine the effects of

acute exposure to a hot and cold environment, without exercise, on gene expression related to mitochondrial development, myogenesis, and proteolysis in human participants. This information is critical for the development of novel approaches to combat mitochondrial and skeletal muscle dysfunction.

METHODS

Subjects

Twelve college-aged males participated in the study. This study was approved by the University Institutional Review Board and all participants were informed of the procedures and any associated risks before signing a written informed consent. Participants came into the laboratory after an overnight fast and having abstained from unaccustomed exercise during the previous 48 h prior. Participants kept an exercise record for two days prior and a dietary record for 24 h before the initial trial. These records were repeated before each subsequent trial. Subjects were tested on 3 separate occasions. Trials were completed in a repeated measure, randomized, and counterbalanced structure. Trials consisted of 3 h of sitting in an environmentally controlled chamber (Darwin Chambers Company, St. Louis, MO) at three different temperatures (33 °C, 7 °C, and 20 °C) each at 60% relative humidity. The temperatures were determined in order to make a direct comparison to a previous study including the exercise stimulus (Heesch et al. 2016). Each trial was separated by approximately one week. All participants were tested at the same time of day. During the experimental trials the participants wore standardized shorts and t-shirts. In addition, the participants were not provided a towel to wipe perspiration. Average monthly temperature for the testing period (April–May) was 56.8°F. Participants tested were natives of the area, therefore not recently exposed to prolonged periods of hot or cold climates.

Muscle Biopsies

Muscle biopsies were taken before and after 3 h exposure to cold, hot, or room temperature conditions. Biopsies were obtained from the *vastus lateralis* muscle using a 5 mm Bergstrom percutaneous muscle biopsy needle with the aid of suction. Each subsequent biopsy during a trial was obtained from the same leg using a separate incision 2 cm proximal to the previous biopsy. The order of the leg biopsied was randomized and the opposite leg was biopsied in the subsequent trial. To account for the heterogeneous nature of the *vastus lateralis* and the effect of testing different legs and locations within the muscle, data was normalized to room temperature condition at the 3 h time-point. Furthermore this approach accounted for the effect of time and/or the impact of the pre-biopsy on the 3 h time-point. After excess blood, connective tissue, and fat were quickly removed, the tissue samples were immersed in RNAlater solution (Qiagen, Valencia CA) and stored at 4 °C overnight, followed by storage at –80 °C for later analysis.

mRNA Extraction

A 19.6 ± 2.9 mg piece of skeletal muscle was homogenized in 800 μ L of Trizol (Invitrogen, Carlsbad CA) using an electric tissue disruptor (Power Gen 125, Fisher Scientific, Pittsburgh PA). Samples were then incubated at room temperature for 5 min, after which 160 μ L of chloroform was added. Tubes were shaken vigorously by hand for 15 s. After an additional

incubation at room temperature for 2 to 3 min, the samples were centrifuged at 12,000 g for 15 min and the aqueous phase was transferred to a fresh 1.5 mL tube. Next, mRNA was precipitated by adding 400 μ L of isopropyl alcohol and samples were incubated overnight at -20°C . The next morning, samples were centrifuged at 12,000 g for 10 min at 4°C and the mRNA was washed by removing the supernatant and adding 800 μ L of 75% ethanol. Samples were vortexed and centrifuged at 7,500 g for 5 min at 4°C . The supernatant was removed, and the dried mRNA pellet was re-dissolved in 100 μ L of RNase-free water. The mRNA was further purified using an RNeasy mini kit (Qiagen) according to the manufacturer's instructions using the additional DNase digestion step (RNase-free DNase set, Qiagen). RNA was then quantified using a nano-spectrophotometer (nano-drop ND-2000, Thermo Scientific, Wilmington, DE). The RNA integrity of the samples was assessed using an Agilent RNA 6000 Kit and a 2100 Bioanalyzer (both from Agilent Technologies, Santa Clara, CA) according to manufacturer's instructions. These quality control measures confirmed the isolation of pure, intact mRNA (260:280 ratio = 1.97 ± 0.15 ; 260:230 ratio = 1.13 ± 0.56 ; RNA Integrity Number = 8.6 ± 0.9).

cDNA Synthesis

First-strand cDNA synthesis was achieved using the Superscript III-first-strand synthesis system for RT-PCR kit (Invitrogen, Carlsbad CA) according to the manufacturer's instructions. The resulting cDNA was then diluted with the appropriate amount of RNase free water to achieve a final cDNA concentration of 0.5 $\mu\text{g}/\mu\text{L}$ in the PCR reaction.

qRT-PCR

Each 20 μL reaction volume contained 1 μL of probe and primer mix (PrimeTime qPCR assay, Integrated DNA Technologies, Coralville, IA), 10 μL Brilliant III Ultra-Fast QPCR Master Mix (Agilent Technologies), 0.3 μL of dye mixture, 6.2 μL of water, and 2.5 μL of sample cDNA. PCR was then run in duplicate on a Stratagene mx3005p PCR system (Agilent Technologies) and a 2-step Roche protocol (1 cycle at 95°C for 3 min, followed by 40 cycles at 95°C for 5 s followed by 60°C for 20 s).

Quantification of mRNA for genes of interest was done using the $2^{-\text{CT}}$ method (Livak and Schmittgen 2001) for muscle samples taken before and 3 h post-temperature exposure. In addition, same method was used to quantify mRNA for genes of interest for muscle samples taken 3 h post-temperature exposure and compared to the room temperature control. For each participant, the geometric mean of the most stable reference genes- beta-actin (ACTB), beta-2-microglobulin (B2M), cyclophilin (CYC), ribosomal protein S18 (RPS18), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was determined by qbase+ geNorm software (Biogazelle, Gent, Belgium) and this value was used to calculate the fold changes of the genes of interest. The genes of interest related to mitochondrial function were ERR α , GABPA, MEF2A, NRF1, PGC-1 α , SIRT, TFAM, and VEGF. The genes of interest related to myogenesis were MSTN, MYOG, MYF5, MYF6, and MYOD. The genes of interest related to proteolysis were FOXO3, MuRF1, and atrogin-1. Tables 1, 2, 3, and 4 represent primer sequencing for real-time PCR.

Core Temperature and Whole Body Metabolism

Core body temperature was monitored continuously during the experimental trials. Participants inserted a Jonah Core Body Temperature Capsule (JCBC Hidalgo Limited, Cambridge, UK) via rectal suppository. The validity of utilizing thermistor capsules during resting conditions has been previously established (Engels et al. 2009). The thermistor capsule transmitted information to the EQO2 LifeMonitor Sensor Electronics Module (SEM; Hidalgo Limited) and recorded the core temperature every 15 s throughout the duration of the trial. Expired gases were collected during each experimental trial to analyze oxygen utilization. A flow and gas calibrated metabolic cart was used to collect all gases (ParvoMedics TrueOne Metabolic System, Sandy, Utah). Gases were collected in 5 min intervals every hour during the 3 h temperature exposure. The last 3 min of the 3 temperature exposure collections were averaged to represent the entire exposure period.

Statistical Analyses

Differences in gene expression were analyzed with a repeated measures two-way ANOVA (time x trial). If significance was indicated with the initial analysis, a Fisher's protected least significant difference post-hoc test was used to evaluate where significant differences occurred. A probability of type I error of less than 5% was considered significant ($p < 0.05$). All data were analyzed using the Statistical Package for Social Sciences software (SPSS 23.0, Chicago, IL).

RESULTS

Participants

A total of twelve college-aged male participants were recruited for the purposes of this study (age 26.4 ± 4.9 , height 183.3 ± 4.4 cm, weight 91.1 ± 30.0 kg).

Gene Expression

There was no significant effect of ambient temperature on gene expression of MSTN ($p = 0.987$), MYOG ($p = 0.444$), MYF5 ($p = 0.343$), MYF6 ($p = 0.458$), or MYOD ($p = 0.201$). However, MSTN and MYF5 decreased over the 3 h trial period ($p < 0.001$, $p = 0.003$, respectively) while MYF6 and MYOD increased ($p = 0.026$, $p = 0.004$, respectively; Figure 1.A). Ambient temperature also had no significant effect on gene expression of atrogen-1, MuRF1, or FOXO3 ($p = 0.543$, $p = 0.693$, $p = 0.102$, respectively). However, FOXO3 decreased with time regardless of temperature ($p = 0.004$; Figure 2.A). There was no significant difference in the gene expression of ERR α ($p = 0.665$), GABPA ($p = 0.080$), MEF2A ($p = 0.630$), NRF1 ($p = 0.651$), PGC1 α ($p = 0.612$), or SIRT1 ($p = 0.080$) between hot, cold, or room temperature conditions. TFAM decreased over the 3 h trial period ($p = 0.029$). However, VEGF was higher in the cold compared to hot ($p = 0.027$) and room temperature ($p = 0.060$) conditions; Figure 3. A). In order to account for the effect of time and/or the impact of the pre-biopsy on the 3 h time-point we also expressed the mRNA relative to the room temperature condition at the 3 h time-point. Using this approach, temperature had no effect on VEGF when comparing cold and hot conditions to the room temperature control (Figure 3. B). Furthermore, comparing cold and hot conditions to the

room temperature control confirms no effect of temperature on all other genes; Figures 1. B, 2. B, 3. B.

Core Temperature and Whole Body Metabolism

Average core body temperature over the 3 h time period was higher in hot (37.2 ± 0.1 °C; $p = 0.001$) and cold (37.1 ± 0.1 °C; $p = 0.013$) conditions compared to room temperature (36.9 ± 0.1 °C). Baseline core temperature was 37.2 ± 0.4 °C in hot, 37.0 ± 0.3 °C in cold, and 37.0 ± 0.3 °C in room temperature conditions. Table 5 details the hour by hour core temperature response for the hot, cold, and room temperature trials. Whole body oxygen consumption was higher in the cold (0.52 ± 0.03 L·min⁻¹ $p = 0.001$) condition compared to hot (0.38 ± 0.01 L·min⁻¹) and room temperature (0.35 ± 0.01 L·min⁻¹); Figure 4 represents oxygen consumption averages for each hour

DISCUSSION

It is of great interest to examine the underlying mechanisms of mitochondrial biogenesis and muscle growth as they relate to the development of pathological disorders. The key aim of the present investigation was to determine the impact of environmental temperature on skeletal muscle gene expression in a human model. Although exercise alone is a valuable therapy to stimulate mitochondrial development and muscle growth, the reduced capacity of a sedentary or diseased population to perform adequate exercise illustrates the need for further development of novel therapies to stimulate mitochondrial development and muscle growth. Furthermore, the effects of exercise combined with temperature exposure have been previously demonstrated to be effective in altering gene expression in the human model. The current study using human participants adds to previous work in animal models and provides additional insights into the impact of environmental temperature on mitochondrial development, myogenesis, and proteolysis.

The main finding of this study was that exposure to hot and cold environments resulted in no significant differences in the expression of most mitochondrial, myogenic, or proteolytic genes. These data are in the agreement with previous studies in animal models that support the necessity of exercise as the primary stimulus for alterations in gene expression, with the temperature at which the exercise is performed having an additional modulatory effect (Seebacher and Glanville 2010). Specifically, Seebacher and Glanville (2010) provided evidence in rats that metabolic changes due to cold exposure are critically dependent on the presence of an exercise stimulus. The relative expression of NRF-1 mRNA increased only in rats exposed to both exercise and cold conditions. These animals exercised five days a week at 12 °C. No alteration in gene expression was observed in rats exposed to cold without the exercise stimulus, nor in the group exercising in the warm environment (22 °C).

Furthermore, cold exposure alone did not increase maximum capacity for substrate oxidation in mitochondria, as measured by cytochrome c oxidase and citrate synthase activities. Although Ijiri et al (2009) demonstrated significant increases in mRNA levels of myostatin and PGC-1 α in chicks exposed to cold, the authors did not report daily physical activity of the animals in the experimental design. Thus, it is unknown whether the

alterations in gene expression were due to temperature exposure alone or its synergistic effects with physical activity.

Induction of several transcription factors, of which PGC-1 α plays an overreaching regulatory role, is necessary for coordination of metabolic pathways related to mitochondrial development (Pilegaard et al. 2003). Previously, it was demonstrated that endurance exercise with cold exposure trigger transcriptional pathways associated with mitochondrial biogenesis (Slivka et al. 2012, Slivka et al. 2013). Specifically, endurance exercise with recovery in a cold environment resulted in higher PGC-1 α mRNA, while recovery in a hot environment resulted in lower expression of PGC-1 α mRNA when compared to recovery in room temperature. In the current study, we demonstrate no alterations in PGC-1 α mRNA with temperature exposure alone, thus emphasizing the critical necessity of an exercise stimulus previously demonstrated to induce changes in gene expression related to mitochondrial biogenesis.

Preliminary data from our laboratory indicate that the influence of local muscle temperature on the process of resistance exercise-induced myogenesis and proteolysis has been previously reported (Shute et al. 2015). It appears that local muscle cooling combined with resistance exercise induces proteolysis, therefore hindering muscle growth, when compared with local muscle heating. In the current study, environmental temperature alone did not provide sufficient stimulus to alter myogenic and proteolytic gene expression, indicating that resistance exercise in conjunction with a local heat and cold application may be necessary to induce changes in gene expression.

Although temperature alone was not sufficient to alter gene expression related to myogenesis and proteolysis, our findings demonstrate that changes in several genes were affected by time, regardless of the temperature condition. Specifically, we observed an overall favorable increase in the expression of genes related to muscle growth. Myostatin, a negative regulator of myogenesis (Thomas et al. 2000), decreased during the 3 h trial period. The increase in MYF6 and MYOD further emphasizes the induction of myogenesis with time. MYF6 is a predominant muscle regulatory factor in adult skeletal muscle and functions as a determinant of myogenic lineage early in the process of myogenesis (Hinterberger et al. 1991; Rhodes and Konieczny 1989; Rudnicki et al. 2008), while MYOD stimulates myoblasts to enter differentiation and join the muscle lineage (Zanou and Gailly 2013). Furthermore, FOXO3 mRNA decreased during the 3 h trial period. FOXO3 is a transcription factor that causes marked atrophy in adult skeletal muscle and has been shown to induce the muscle-specific ubiquitin ligase atrogen-1 (Mammucari et al. 2008). Although we demonstrated these changes in gene expression with time, regardless of the temperature condition, it could be argued, that the biopsy procedure itself caused the increased myogenic drive that was observed. The proinflammatory environment following a muscle biopsy coordinates the activity of satellite cells required for muscular regeneration (Souza et al. 2015). Satellite cells are stimulated to aid in muscle recovery by differentiating into myoblasts, thus the myogenic process is tightly bound to the upregulation of specific transcription factors, such as MyoD, MYF5, and MYF6 (Braun et al. 1992; Rudnicki et al. 1992; Thomas et al. 2000). Gene expression of VEGF was higher in the cold compared to hot and room temperature conditions, which is in agreement with other studies. VEGF is a

major regulatory factor in skeletal muscle adaptation to the cold environment, stimulating angiogenesis and thermogenesis (Kim et al. 2005, Fredriksson et al. 2005). Thus, the observation of cold-induced increase in VEGF mRNA may be expected. Additionally, the induction of several genes could be due to the inability to completely control physical activity prior to testing, specifically walking to the laboratory and morning activity. However, the participants were asked to keep an exercise and dietary log to account for any variations in their routine. Although we demonstrate alteration of several genes affected by time, we also expressed mRNA of the cold and hot conditions relative to room temperature at the 3 h time-point. This approach allowed us to better control for the potential influence of the tissue trauma resulting from muscle biopsies and address our purpose of determining the impact of temperature. No differences were observed in the gene expression of any of our genes of interest, which further supports our conclusion that temperature alone isn't sufficient for alterations.

Based on the findings from the current investigation, it is concluded that temperature alone has very minimal effect on alterations in the expression of genes related to mitochondrial development, myogenesis, and proteolysis, despite the differences in oxygen consumption and core body temperature. Recently, exposure to ~73 °C for 30 min resulted in a significant down regulation of genes important for mitochondrial function (Petrie et al. 2016). Incorporating higher environmental temperatures and relative humidity in the study design could induce larger increases in core and muscle temperature necessary for alterations of genes related to mitochondrial development, myogenesis, and proteolysis. This study further confirms data from animal models (Seebacher and Glanville 2010) that physical activity is a necessary stimulus for the alterations in skeletal muscle gene expression observed with temperature manipulation in a human model.

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Figure 1.

A. mRNA expression of myogenic genes normalized to pre-temperature exposure. Values are mean $\pm$ SE. † $p < 0.05$ from pre-temperature exposure mRNA expression. RT = room temperature. (MSTN: myostatin, MYOG: myogenin, MYF5: myogenic regulatory factor 5, MYF6: myogenic regulatory factor 6, MYOD: myogenic differentiation). **B.** mRNA expression of myogenic genes normalized to RT. Values are mean $\pm$ SE.

Figure 2.

A. mRNA expression of proteolytic genes normalized to pre-temperature exposure. Values are mean $\pm$ SE. † $p < 0.05$ from pre-temperature exposure mRNA expression. RT = room temperature. (MuRF1: muscle RING-finger protein 1, FOXO3: forkhead box O3). **B.** mRNA expression of proteolytic genes normalized to RT. Values are mean $\pm$ SE.

Figure 3.

A. mRNA expression of genes related to mitochondrial biogenesis normalized to pre-temperature exposure. Values are mean $\pm$ SE. $\dagger$ $p < 0.05$ from pre-temperature exposure mRNA expression. * $p < 0.05$ significant interaction (cold > hot). RT = room temperature. (ERR α : estrogen related receptor alpha, GABPA: GA binding protein, MEF2A: myocyte enhancer factor 2A, NRF1: nuclear respiratory factor 1, PGC1 α : peroxisome proliferator-activated receptor gamma coactivator 1 alpha, SIRT1: sirtuin 1, TFAM: mitochondrial transcription factor A, VEGF: vascular endothelial growth factor). **B.** mRNA expression of genes related to mitochondrial biogenesis normalized to RT. Values are mean $\pm$ SE.

Figure 4.

Whole body oxygen consumption averages for each hour. Values are mean $\pm$ SE. * $p < 0.05$ from cold; † $p < 0.05$ from hot. RT = room temperature.

Table 1

Primers and probes used for real-time PCR (reference genes).

Gene Symbol	Accession Number	Primer 1	Primer 2	Probe
GAPDH	NM_002046(1)	ACATCGCTCAGACACCATG	TGTAGTTGAGGTCAATGAAGGG	AAGGTCGGAGTCAACGGATTTGGTC
PPIA	NM_021130(1)	CATCCTAAAGCATAACGGGTCC	TCCTTCACTTTGCCAAACACC	TGCTTGCCATCCAACCACTCAGTC
RPS18	NM_022551(1)	GTTCCAGCATATTTGCGAGT	GTCAATGTCTGCTTTCCTCAAC	TCTTCGGCCCACACCCTTAATGG
ACTB	NM_001101(1)	ACAGAGCCTCGCCTTTG	CCTTGACATGCCGGAG	TCATCCATGGTGAGCTGGCGG
B2M	NM_004048(1)	GGACTGGTCTTTCTATCTCTGT	ACCTCCATGATGCTGCTTAC	CCTGCCGTGTGAACCATGTGACT

Table 2

Primers and probes used for real-time PCR (mitochondrial-related genes).

Gene Symbol	Accession Number	Primer 1	Primer 2	Probe
PGC1-α	NM_013261(1)	GAGTCTGTATGGAGTGACATCG	TGTCTGTATCCAAGTCGTTTAC	ACCAGCCTCTTTGCCAGATCTTC
ESSRα	NM_004451(1)	CTATGGTGTGGCATCCTGTG	TCTCCGCTTGGTGATCTCA	TGGTCCTCTTGAAGAAGGCTTTGCA
GABPA	NM_002040(2)	TGGAACAGAGAAAGCAGAGTG	TGTAGTCTTGGTTCTAGCAGTTTC	TGGTTCATTGATGTCTATGGCCTGGC
NRF1	NM_005011(2)	GATGCTTCAGAATTGCCAACC	GTCATCTCACCTCCCTGTAAC	ATGGAGAGGTGGAACAAAATTGGGC
TFAM	NR_073073(3)	TGGGAAGGTCTGGAGCA	GCCAAGACAGATGAAAACCAC	CGCTCCCCCTTCAGTTTTGTGTATTT
VEGF	NM_001025367(18)	CCATGAACCTTTCTGCTGTCTTG	GCGCTGATAGACATCCATGA	TGCTCTACCTCCACCATGCCAAG
MEF2A	NM_001171894(5)	TCCTCAGAGACCACCAAGT	GTTTACAAATCCATTCCCCACTG	TCAACATCCCACCTGCATTGCC
SIRT1	NM_001142498(2)	TTCTTTTGCAACAGCATCTTG	GTTTCATGATAGCAAGCGGTTTC	CTCGATGTCCTAGGTGCCCAGC

Note: PGC1- α : peroxisome proliferator-activated receptor gamma coactivator 1 alpha, ESSR α : estrogen related receptor alpha, GABPA: GA binding protein, NRF1: nuclear respiratory factor 1, TFAM: mitochondrial transcription factor A, VEGF: vascular endothelial growth factor, MEF2A: myocyte enhancer factor 2A, SIRT1: sirtuin 1.

Table 3

Primers and probes used for real-time PCR (proteolytic genes).

Gene Symbol	Accession Number	Primer 1	Primer 2	Probe
Atrogin-1	NM_001242463(3)	TCAGCCTCTGCATGATGTTC	CAACAGACTGGACTTCTCAACT	CACTGACCTGCCTTTGTGCCTACA
MuRF-1	NM_032588(1)	GCAACTCACTTTTCTTCTCATCC	TGCAGACCATCATCACTCAG	ACCTGGTGACTGTTCTCCTTGGTC
FOXO3	NM_001455(1)	ATTCTGGACCCGCATGAATC	CGTGCCCTACTTCAAGGATAAG	AGGTTGTGCCGGATGGAGTTCTTC

Note: MuRF1: muscle RING-finger protein-1, FOXO3: forkhead box O3

Table 4

Primers and probes used for real-time PCR (myogenic genes).

Gene Symbol	Accession Number	Primer 1	Primer 2	Probe
MYOD	NM_002478(1)	TGCTGGACAGGCAGTCTA	CTCCGACGGCATGATGG	TCGACACCGCCGCACTCTTC
MYF5	NM_005593(1)	GGCATATACATTTGATACATCAGGAC	CACCTCCAAGTCTCTGATG	TGCTGTCAAAAGTACTGCTCTTTCTGGA
MYF6	NM_002469(1)	CTACTCGAGGCTGACGAATC	CAGCTACAGACCCAAACAAGA	TGATAACGGCTAAGGAAGGAGGAGCA
MYOG	NM_002479(1)	AGAAGTAGTGGCATCTGTGG	GACAGCATCACAGTGAAGA	ATGCCCGGCTTGAAGACAATCT
MSTN	NM_005259(1)	TCGTGATTCTGTTGAGTGCT	TGTAACCTTCCCAGGACCA	TCTTTTGGTGTGTCTGTTACCTTGACCT

Note: MYOD: myogenic differentiation, MYF5: myogenic factor 5, MYF6: myogenic factor 6, MYOG: myogenin, MSTN: myostatin.

Table 5

Core Temperature (°C)

	Hour 1	Hour 2	Hour 3
Cold	37.2 ± 0.1 *	37.0 ± 0.1 *	37.0 ± 0.1 *
Hot	37.0 ± 0.1 †	37.1 ± 0.1 *†	37.3 ± 0.1 *†
Room Temperature	37.0 ± 0.1	36.9 ± 0.1	36.7 ± 0.1

Note: Data are mean ± SE

*
 $p < 0.05$ different from room temperature;†
 $p < 0.05$ from cold (main effects of temperature).