Study Protocol and Statistical Analysis Plan

Title: Impact of muscle temperature on muscle growth and breakdown: Cooling during resistance training

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Abstract

The purpose of this study is to examine the effect of human skeletal muscle temperature during resistance exercise on myogenic and proteolytic signaling. Study participants will be eligible if they are male or female aged 19-45, recreationally active, and considered 'low risk' for activity participation. Participants will perform bilateral resistance exercise bouts at an intensity that should stimulate a muscle growth response in the Vastus Lateralis. During the resistance exercise, the participants will receive a cold (10°C) intervention on the experimental limb, while receiving a neutral temperature intervention (22°C) on the control limb. Skin and intramuscular temperatures, local blood flow, and skeletal muscle biopsies will be collected from each leg pre-, immediately post, and following the 3-hour recovery to evaluate myogenic and proteolytic related gene and protein expression.

Purpose and Rationale

To test the hypotheses that skeletal muscle cooling will inhibit the cell signaling response associated with muscle growth and that skeletal muscle cooling will inhibit the resistance exercise induced skeletal muscle growth response.

Maintaining an adequate muscle mass is critical for health and athletic performance. The mechanisms regulating muscle mass are widely studied and are of interest to a diverse group of people, including athletes, strength and conditioning coaches, athletic trainers, and health care professionals. Skeletal muscle is a highly plastic tissue able to adapt to changes in response to various stimuli, such as mechanical stress, physical activity, nutrients, and growth factors (7). Increased load on muscle results in an increase in its mass or hypertrophy, whereas unloading or disuse leads to a decrease in mass or atrophy. Based on the currently known mechanisms of maintaining and increasing skeletal muscle mass, resistance training is considered to be a powerful stimulus of muscle hypertrophy. It has been consistently demonstrated (12-15) that a single bout of resistance exercise is sufficient to enhance protein synthesis, which in turn may stimulate myogenic pathways in skeletal muscle. Furthermore, resistance exercise induces translational changes in the muscle, which may serve as the basic regulatory mechanism in hypertrophy (16). Protein synthesis and protein breakdown are regulated through multiple signaling pathways, including the Akt/mTORC1 pathway. In this pathway, Akt activation by stimuli, such as mechanical stress can lead to the activation of mammalian target of rapamycin (mTOR). mTOR is a serine/threonine kinase that plays a key role in various biosynthetic processes, including protein synthesis, cell growth, and cell proliferation (17-20). During growth factor and nutrient sufficiency, mTORC1 phosphorylates the translational regulator p70 ribosomal protein S6 kinase 1 (S6K1). Phosphorylation activates p70 S6K1 and allows it to phosphorylate the S6 ribosomal protein, directly leading to protein synthesis at the ribosome (21). p70 S6K1 phosphorylation is a good acute marker of muscle protein synthesis, as it is highly positively correlated with increased muscle mass following six weeks of electrical stimulation in rodent hindlimbs (22) and 12 weeks of resistance training in humans (23). In a non-phosphorylated state, 4E-BP1 binds to eukaryotic translation initiation factor 4 (EIF4) and inhibits its ability to bind the 40S ribosomal subunit. Phosphorylation of 4E-BP1 leads to its dissociation from EIF4, resulting in the 40S ribosomal subunit activating and initiating mRNA translation (24). In addition to regulating protein synthesis, mTORC1-mediated phosphorylation of S6K1 and 4EBP also promotes cell growth and cell cycle progression (25, 26).

Even though resistance exercise is a powerful stimulator of muscle synthesis, it also inevitably stimulates protein degradation (12). Maintenance of skeletal muscle depends upon the balance of protein synthesis and degradation, and it plays a crucial role in the remodeling of muscle tissue. Muscle protein degradation is modulated by forkhead box O3 (FOXO3a), a transcription factor responsible for the expression of proteolytic genes, mainly atrogin-1 and MuRF1 (27, 28). FOXO3a is targeted for phosphorylation by several protein kinases, including AKT. When phosphorylated, FOXO3a is translocated out of the nucleus, and cannot exert its pro-transcriptional effect on aforementioned proteolytic genes (29).

Individuals with compromised muscle mass appear to have a similar response to resistance exercise compared to healthy controls based on mRNA expression of muscle regulatory factors (30). However, there appears to be a lower level of muscle regulatory factor protein accumulation in aged muscle tissue following resistance exercise (31), as well as a lower capacity for hypertrophy in those with muscle wasting (31-33). It has been demonstrated that individuals with compromised muscle mass have a similar myogenic response at the transcriptional level, compared to healthy controls (30). However, there appears to be a lower level of protein accumulation in aging individuals, as well as a lower hypertrophic response to resistance exercise in individuals with muscle wasting. These observations lead to a conclusion that the overall limitation in myogenic response may result from differences in translational and post translational signaling. These findings indicate that these individuals may have a limited overall myogenic response to contractile stimuli, possibly due to differences in translational and post-translational signaling. These findings highlight the need for development of novel therapies to treat the loss of muscle mass and/or increase hypertrophy above and beyond traditional resistance training. These exploratory studies propose the novel use of local muscle temperature manipulation to augment resistance exercise-induced myogenesis in humans. Previously, altered temperature resulted in stimulation of Akt/mTOR signaling in rodents and human cell cultures and downregulated the activity of FOXO3a (7, 34, 35), however the responses in exercising human muscle are currently unknown and are to be explored in the proposed study. Furthermore, a more comprehensive view of myogenic and proteolytic processes following resistance exercise will be examined through the utilization of translational and post-translational markers.

The investigator's laboratory has recently published a proof-of-concept study that investigated the human myogenic and proteolytic transcriptional response to resistance exercise when one leg was heated, and the other leg was cooled (5). The results from this study indicate that the exercise-induced response was different between the heated and cooled leg, in favor of the heated leg. With this initial study design, it cannot be determined if heating produced a favorable response or if cooling produced a inhibitory response. Furthermore, it is unsure the impact of temperature independent of exercise. The currently proposed studies will allow these questions to be answered with greater mechanistic resolution. There are few human studies that have addressed this issue. However, these studies do not lead to the same conclusions. No statistical benefit in muscle hypertrophy or strength was observed with local muscle heating after 12 weeks of resistance training (Stadnyk 2018). It should be noted that the difference in muscle temperature between experimental and control conditions was only ~1°C. Using a similar design Goto et al (2007) demonstrated an increase in muscle hypertrophy and strength, although this observation may be due the non-dominant limb serving as the experimental (heating) condition. Unfortunately, these previous studies did not measure any molecular aspects that could have allowed more clear interpretation of the data and assessment of the protocols used nor did they investigate the impact of heat alone. To our knowledge only one study has investigated the resistance exercise mTOR signaling response in humans. This study indicates that heat enhances mTOR signaling after resistance exercise in human skeletal muscle (Kakigi 2011). Unfortunately, this study used

microwave therapy to increase the muscle temperature much quicker and greater than typical external heat modalities. The issue with the use of microwave therapy is that this modality may have other non-thermal effects on cell membrane integrity (Rougier 2014) which in turn could activate the mTOR pathway. More clear rationale for the use of heat to enhance muscle growth comes from rodent and culture models. Heat stress appears to activate the Akt/mTOR pathway (Yoshihara, 2013) and attenuate muscle atrophy (Naito, 2000) in rats.

The use of cold-water immersion has become a popular post-exercise recovery strategy despite a lack of evidence of its efficacy. Indeed, much of the data available of the human skeletal muscle response to local cold has used a cold-water immersion protocol. Strength training adaptations appear to be blunted when incorporating cold water immersion (36), which may be due to suppressed ribosome biogenesis (37) and anabolic signaling (38). Low cell culture temperature inhibits myogenic differentiation (39), and anabolic response (10, 40). These results are not without controversy as other applied studies and subsequent meta-analysis have shown a mild benefit of cold interventions (41). The current studies will help understand the disconnect between applied human model studies and mechanistic studies in rodents and cell culture by implementing molecular cell-signaling data into our human model.

Investigator's previous work and the foundational studies presented above will allow us to take a strategic approach and build off the previous work. We will take applied study design cues from the previous human studies and incorporate mechanistic components from rodent and culture studies. Furthermore, the studies proposed here will be able to determine the potential separate effects of temperature and those of effects of temperature only observable in the presence of exercise.

Participants

12 participants, age 19 to 45, will need to complete the study in order to achieve the scientific objectives of the research. Keeping an age range of 19-45 ensures that the participants will be relatively homogenous in terms of overall health and thus not present added risk.

Research Plan

A total of 2 visits to the laboratory will be required following the signing of informed consent. Given participant consent, photographs may be taken during the trials to document the research and use in future presentations.

Initial Visit (~1.5-2 hours):

Participants will complete a risk stratification form based on ACSM guidelines and must be considered low risk in order to participate in this study. Height and weight will be measured using a medical scale. Body fat will be assessed with hydrostatic weighing using an electronic load cell-based system (Exertech, Dresbach, MN) correcting for residual lung volume, or BIA body composition analyzer (InBodyUSA, Cerritos, CA) if needed.

Participants will then perform a Repetition Max Test for bilateral seated leg press and seated leg extension using the following protocol on isotonic exercise machines at a weight designed to create failure between 8 and 12 repetitions. Following a short warm-up on a cycle ergometer, the participants proceed with a bilateral leg press warm-up consisting of one set of ten repetitions at a self-determined light load, then one set of 5 repetition at a self- determined moderate load, and one set of 1 repetition at a predicted 12 rep maximum as predicted in relation to their Fat Free Mass. During the warm-up participants will be coached on form and observed for their ability to complete the repetition max test. Weight for the test will be altered to suit the participants' ability to complete approximately 12 repetitions. Once the desired weight is determined,

participants will be asked to complete as many repetitions as possible until the participant can no longer complete a repetition using a full range of motion.

An identical testing protocol is then performed for the leg extension. This data will be used to estimate a 12RM workload, that would be appropriate for hypertrophic growth using the Watham equation (46).

Experimental Visit (~6.5-8 hours):

Participants will be instructed to arrive to the lab having fasted overnight, and they will be asked to avoid strenuous activity, alcohol consumption, tobacco use, and drug use for the 24-hour period leading up to experimental trial.

Participants will complete the following experimental protocol briefly described: consume a small meal, a pre-exercise muscle biopsy, precool for 30 minutes, complete the exercise intervention, immediately post-exercise biopsy, 4-hour recovery with cooling, and post- recovery biopsy. At three time points (pre-exercise, immediately post-exercise, and post-4-hour recovery), skin temperature, muscle temperature, blood flow, and muscle tissue biopsies will be taken.

Details are as followed:

Meal

When participants arrive, a consistent meal will be provided to standardize dietary intake. Food choices will be given to participants to create a meal between 700-800 calories, containing approximately 25-35 g of protein, 75-85 g of carbohydrates, and 35-45 g of fat. These foods will be made-up of commercially available pre-packaged foods. They will have approximately 30 minutes to consume their meal.

Cooling protocol

Participants will have Game Ready Med 4 elite (https://gameready.com/med-4-elite-contrast- therapy-unit/.) thigh wraps around each thigh. This system circulates cooled liquid through a wrap specifically fitted for different body segments. One thigh will be cooled while the other thigh will be wrapped with the cuff, but not have any liquid circulating through it. These wraps do provide slight compression and thus it is important to apply the wraps to both legs. Each leg is assigned to either cold experimental group or control, will be randomized and counter-balanced between participants. The liquid temperature circulating through the thermal wraps will be set to 10°C. This temperature has been shown in investigator's previous works to not injure the participants' skin. The thermal wraps will be worn during a 30-minute cool down period prior to the exercise bout, during the exercise intervention, and during a 4-hour recovery period.

Exercise Intervention

The participants will complete an intervention designed to stimulate the muscle growth signaling pathway (hypertrophy) in the Vastus Lateralis (outer thigh), via bilateral seated leg press and bilateral seated leg extension on isotonic exercise machines. Participants will perform four sets of bilateral leg press exercises at the weight determined to be their 12 RM from the repetition max test. This should allow completion to failure of 8-12 repetitions. So, if more than 12 reps are able to be completed, weight will be added to the resistance for the subsequent sets. Conversely, if less than 8 reps lead to failure, the weight should be reduced for the subsequent sets. Constant dialogue with participants will give investigators the ability to monitor set weights to maximize a hypertrophic response. Participants will be allowed 2 minutes of rest between each set. Upon

completion of 4 sets, the participants will be given 5 minutes of rest prior to repeating this protocol for bilateral leg extensions.

Biopsies

A total of 6 muscle biopsies (1 per leg) will be obtained from the vastus lateralis (outer thigh) muscle. One from each leg for each of the three collection time-points, pre-exercise, immediately post-exercise, and post-4-hour recovery. The PI will conduct these biopsies, having successfully completed over 600 biopsies without any adverse events. Briefly, the area above the vastus lateralis muscle belly will be shaved and cleaned with alcohol. 1% xylocaine is then injected under the skin with a 25 ga hypodermic needle. The Xylocaine will be ordered with a prescription provided by the participating physician.

The area is then further disinfected with betadine, and a sterile fenestrated drape is placed over the biopsy site. Using sterile techniques, a small incision is made through the skin (~5mm) and the Bergstrom biopsy needle is inserted through the incision into the belly of the muscle. The Bergstrom needle then takes a small clip of muscle (~50mg) and is removed from the muscle and stored in a chemical stabilizer at -30ºC. Slight pressure is held over the incision for approximately 2 minutes and is then closed using steri-strips and a band-aid with antibiotic ointment. The leg is further dressed with a compression bandage.

Participants are all instructed how to care for the wound and given the investigators contact information in case they have further questions.

Intramuscular Temperature
Using the incision from the muscle biopsy, a hypodermic (~26ga) thermocouple (Physitemp Instruments LLC, Clifton, NJ) that is smaller than the needle used to inject the xylocaine will be inserted into the belly of the muscle to measure intramuscular temperature. The thermocouple readings stabilize in approximately 3 seconds and the thermocouple is then removed. Under normal circumstances, the sensation of this temperature reading is similar to an intramuscular injection. However, with the use of the biopsy incision as the insertion point there is very little sensation associated with this measure. Recordings for intramuscular temperature will be collected during the biopsy process following the 4-hour temperature intervention.

Skin Temperature
Skin Temperature will be measured on the surface of each thigh using a skin thermistor (Physitemp Instruments LLC, Clifton, NJ), infrared digital thermometer, and/or using an infrared thermal camera. Measurements will be taken at the same time as the intramuscular. The thermistor is simply placed on the skin for approximately 3 seconds and is then recorded. The infrared digital thermometer can measure and record surface temperature from a distance of 12 inches. The infrared thermal camera can measure and record surface temperatures for a field of multiple surfaces.

Gene Expression Analysis
The muscle tissue collected will be processed and analyzed for transcriptional changes of genes associated with muscle growth and breakdown. Muscle mRNA content will be evaluated using real-time reverse transcription quantitative polymerase chain reaction (real- time RT-qPCR). Quantification of mRNA for genes of interest will be computed using the 2-△△CT method and compared to stable reference genes. Probes and Primers that will target the specific gene sequences will be attained from Integrated DNA Technologies (IDT, Coralville, IA).

Protein Expression Analysis

The muscle tissue collected will be processed and analyzed for proteins associated with muscle growth and breakdown. Standard western blotting protocols will be optimized and used to evaluate the muscle growth signaling cascade, using antibodies specific to the proteins of interest and normalized to total protein.

Statistical Analysis

Recruitment

Previous exercise research suggests that the difference in response of matched pairs is normally distributed with a standard deviation of 0.86. If the true difference of the mean response of matched pairs is 0.75, we will need to complete 12 experimental trials for each condition to be able to reject the null hypothesis that this response difference is zero with a power of 0.8. The Type I error probability associated with this test of the null hypothesis is 0.05.

Interventions

A repeated measures two-way ANOVA will be used for the dependent variables. If the F-ratio values are found to be significant then a Fishers protected Least Significant Difference post hoc will be performed to evaluate where the significance occurs. The probability of type I error must be less than 5% in order to be considered significant ($p < 0.05$). All Statistical data will be analyzed using the Statistical Package for Social Sciences software (SPSS 24.0)