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mTOR Pathway Activation Following Resistance Exercise with Vibration in Human Subjects

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ABSTRACT

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Functional adaptations in human skeletal muscle following a period of resistance exercise are the result of regular activation of cellular signaling pathways that elevate muscle protein synthesis. It has been reported that the addition of whole body vibration (WBV) to a resistance exercise program enhances performance. Such improvements in muscle function may be the result of increased activation of cellular signaling pathways associated with muscle growth. Purpose: We have investigated whether an acute bout of resistance exercise in combination with WBV results in a greater activation of the mTOR signaling pathway compared to resistance exercise alone. Methods: Eight untrained college-age males (23 ± 2 yrs, 179 ± 1 cm, 75.0 ± 2.5 kg, and 12.6 ± 1.8% body fat) performed unilateral leg press exercises with (Vbx) and without (RT) vibration. Muscle samples were obtained from the vastus lateralis muscle pre-exercise (baseline) and one-hour following the bout of resistance exercise. Muscle tissue samples were analyzed for phosphorylated levels of mTOR, p70S6K, and 4E-BP1 proteins. Results: One-hour following the resistance exercise bout there were no differences between phosphorylated levels of mTOR or 4E-BP1 in Vbx or RT ($p > 0.05$). Levels of phosphorylated p70S6K were increased at the one-hour post-exercise time-point in both Vbx (baseline: 504 ± 286 OD; post: 5039 ± 2351 OD, $p < 0.05$) and RT (baseline: 356 ± 131 OD; post: 5430 ± 1218 OD, $p < 0.05$); however, there was no difference in protein phosphorylation levels between conditions ($p > 0.05$). Conclusion: Vibration does not augment acute activation of the mTOR signaling pathway in human skeletal muscle suggesting that performance benefits resulting from combining resistance exercise and vibration may not be the result of an enhanced cellular growth response.

Keywords: mTOR, p70S6K, 4E-BP1, muscle growth, signaling, whole body vibration

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Introduction

Mechanical overload of human skeletal muscle results in increased muscle size and functional capacity (29). These adaptations have their bases in the activation of cellular signaling pathways and the subsequent increase in protein synthesis (6, 7, 15, 43). In an attempt to augment muscle adaptation and function, whole body vibration (WBV) has become a popular tool used in conjunction with resistance exercise (28, 32, 39-41). Although data exist that support the acute benefits of WBV on muscle function, little information is available regarding the impact of WBV on the cellular responses to exercise in skeletal muscle (34, 36, 44).

Muscle strength (3, 27, 28, 31, 32, 39-41) and power (10, 11, 28, 32, 39, 41) have been shown to improve when combining various vibration treatments with resistance exercise. These positive effects of vibration treatment have been seen with subjects exercising on WBV platforms (3, 10, 11, 31, 39-41), with vibrating cables (27, 28), and with other specialized vibration exercise equipment (32). The enhanced benefits from vibration have been seen in untrained (10, 11, 32, 39, 40) and trained (27, 28, 39-41) adults as well as in older individuals (3, 31). The application of vibration is also effective at improving whole muscle function at low and high intensities of exercise (32).

Vibration has been associated with enhanced cellular effects in models such as mouse myoblast cells (44), tendon vibration with hindlimb unloading in rats (21), WBV during long-term bed rest in humans (33, 34, 38), and local vibration in human skeletal muscle (36). The mechanism of these cellular effects has not been elucidated. However, it has been reported that myotube formation with vibration was suppressed with administration of a PI3K inhibitor (44); a route by which mTOR is activated. This association implicates the mammalian target of rapamycin (mTOR) signaling pathway (44).

Vibration treatments have demonstrated physiological effects and vibration has been linked to a skeletal muscle growth signaling pathway (36, 44). mTOR has been extensively studied for its part in mediating skeletal muscle growth through activation of protein synthesis in rodent and human models (19). Human research has shown that resistance exercise can independently activate the mTOR signaling pathway (8, 15, 16, 19). In this study, we determined whether WBV combined with resistance exercise would augment the activation of the mTOR pathway compared to resistance exercise alone. We hypothesized that the addition of WBV during resistance exercise would enhance the acute activation of the mTOR pathway.

Methods

Experimental Design

This study explored the acute effects of a single session of submaximal resistance exercise with and without whole body vibration (WBV) on skeletal muscle hypertrophy signaling through the mTOR signaling pathway. Subjects performed unilateral leg press exercises with (Vbx) and without (RT) WBV. The exercise condition was counter-balanced. Vibration was administered through a WBV platform (VibePlate 2424, Adrian, MI, USA) integrated onto a seated leg press foot-plate (Cybex Seated Leg Press, Cybex International, Medway, MA, USA) (Figure 1). The frequency (40 Hz) and amplitude (~2 mm) of the vertical vibration platform integrated on the leg press was tested with four high-speed video cameras (250 Hz, VICON, Santa Rosa, CA, USA).

Subjects

We studied 8 untrained, healthy male subjects (mean ± SE: 23 ± 2 yrs, 179 ± 1 cm, 75.0 ± 2.5 kg, and 12.6 ± 1.8% body fat) who were physically active but were not currently engaged in

a regular exercise program. Subjects were informed of the study procedures and screened for qualifications (height, weight, percent body fat, physical activity patterns, and exercise routines). All subjects that qualified gave informed written consent before participating in the study which was approved by the Institutional Review Board of Brigham Young University (which is in compliance with the *Declaration of Helsinki*). Each subject provided a brief history of their recent normal activity. Subjects were instructed to maintain normal activity levels and not increase physical activity (e.g. exercise) for at least 48-hours prior to participating in the study (12, 15, 16, 19).

Procedures

The day prior to the exercise session, the subjects were given a standardized meal (62% carbohydrate, 17% fat, and 21% protein, 12 kcal/kg) and snack to consume at 18:00 and 22:00 hours, respectively (meal and snack: 60% carbohydrate, 19% fat, and 21% protein, 15 kcal/kg). The subjects were instructed not to eat anything else until the completion of the exercise session the following day. The snack was given to avoid a prolonged fast during the study (15, 16, 19).

On the morning of the exercise session, subjects arrived at the Human Performance Research Center at 09:00 hours to be weighed and prepped for pre-exercise (baseline) muscle biopsy. Muscle biopsy samples were taken from the lateral portion of the vastus lateralis (VL) of both legs with a 5 mm Bergström biopsy needle. The biopsy site was between 15-25 cm from the mid patella (15, 19). The location of the biopsy site was shaved, disinfected with an antiseptic solution and anesthetized with 3 cc of 1% lidocaine (with epinephrine). The harvested muscle tissue was immediately cleaned of blood and connective tissue, then snap frozen in liquid nitrogen and stored at -80°C until analysis.

Thirty minutes following the baseline muscle biopsy, subjects warmed up each leg individually with one set of 10 repetitions without vibration at 30% of their individual body weight (BW). Following the warm-up, subjects performed 4 sets of 10 at 60% BW with three-minute rest periods between sets for each leg. The Vbx leg performed the leg press exercise concurrent with WBV at frequency of 40 Hz and an amplitude of 2 mm (24). Subjects performed each repetition at a consistent rate (~3 seconds per repetition) with their arms across their chest and their foot placed in the middle portion of the WBV platform. The test administrator instructed subjects to push with a flat foot on the platform.

Upon the completion of the leg press exercises, subjects rested for one hour and then underwent the post-exercise muscle biopsy in each leg. The post-exercise tissue samples were taken through the same incision on each leg with the biopsy needle inserted at a different angle so that each biopsy sample will be taken approximately 2 cm apart.

Tissue Preparation

Muscle tissue samples were ground glass homogenized in chilled tubes in a 10% w/v dilution (50 mM Tris-HCl, (pH 7.4), 250 mM mannitol, 50 mM NaF, 5 mM Sodium Pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM DTT, 1 mM benzamidine, 0.1 mM phenylmethane sulfonyl fluoride, 5 µg/ml soybean trypsin inhibitor). Homogenized samples underwent three freeze-thaw cycles and were then clarified by centrifugation at 16,000 g for 10 minutes at 4°C. Supernatant was collected and transferred into fresh tubes and stored at -80°C until further analysis. Total protein concentrations were determined in triplicate using the DC Protein Assay (Bio-Rad, Hercules, CA, USA). Sample and standard absorbances were measured at 750 nm on a micro plate reader (VICTOR3, PerkinElmer Life and Analytical Sciences, Shelton, CT, USA).

Immunoblotting

After combining with a 2x loading buffer (125 mM Tris (pH 6.8), 4% SDS (sodium dodecyl polyacrylamide), 20% glycerol, 5% β-mercaptoethanol (BME), 0.01% bromophenol blue) tissue samples homogenates (20 μg total protein) were loaded in duplicate and separated via electrophoresis (Mini Protean II, Bio-Rad Laboratories, Hercules, CA, USA) at constant voltage (200 V). Polyacrylamide gel percentage was optimized for the protein of interest (mTOR, 5%; p70S6K, 7.5%; 4E-BP1, 15%). Following electrophoresis, proteins were transferred to polyvinylidene difluoride membrane (PVDF) (Bio-Rad, Hercules, CA, USA) for 120 minutes at constant current of 250 mA. Following protein transfer, the membranes were placed in 5% milk blocking solution for one-hour at room temperature with gentle rotation. The membranes were rinsed with TBST solution (1X Tris-Buffered Saline Tween-20) four times for 5-minutes at room temperature. Membranes were then incubated with the appropriate primary antibody diluted in 5% bovine serum albumin (BSA) solution overnight at 4°C with constant rocking (mTOR (Ser2481; 1:1000; #2983), phospho-mTOR (Ser2448; 1:500; #2971), p70S6K (1:1000; #9202), phospho-p70S6K (Thr389; 1:500; #9205), 4E-BP1 (Ser112; 1:1000; #9644), phospho-4E-BP1 (Thr37/46; 1:500; #9459) (Cell Signaling Technology, Danvars, MA, USA)). Membranes were rinsed (4×5 min) then underwent secondary antibody incubation for 60 minutes at room temperature in 1% milk (Anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody, Cell Signaling Technology (1:2000; #7074)). Follow rinses (4×5 min), membranes were treated for 5-minutes with a chemiluminescent solution (Immun-Star™ WesternC™ Chemiluminescnece (170-5070), Bio-Rad, Hercules, CA, USA). Digital images were captured with a CCD camera mounted on a ChemiDoc XRS imaging system (Bio-Rad, Hercules, CA,

USA) and analyzed with Quantity One 1-D Analysis Software (Version 4.6.5, Bio-Rad, Hercules, CA, USA).

Statistical Analysis

All analyses were made with JMP 10 (SAS Inc., Cary, NC, USA). Comparisons were made using a 2 × 2 repeated-measures analysis of variance (ANOVA) to detect the differences in the conditions between time points. Pre-exercise levels (baseline) were used as a covariate during the analysis for mTOR as there were differences between the conditions at baseline ($F_{1,13}$=101.1, $p<0.0001$). A logarithmic transformation of p70S6K was utilized in order to meet the assumptions associated with an ANOVA analysis. Tukey HSD post hoc tests was used to determine the activation of the signaling (mTOR, p70S6K and 4E-BP1) within each condition. The dependent variables were mTOR, p70S6K and 4E-BP1 and the independent variables were condition and time point. Significance was set at $p < 0.05$. All values are expressed as mean ± S.E.

Results

Exercise Performance

All subjects completed the exercise session of 4 sets of 10 repetitions without adjusting weight, number of repetitions, or rest period. The average exercise load was 45.7 ± 1.5 kg which represented 61.0 ± 0.2% of subject body weight. Subjects performed the exercise sets at a rate of 28 ± 1 seconds per set. The ability to perform unilateral leg press exercises was not different between left and right legs.

Signaling

mTOR phosphorylation (Figure 2). There was not a significant difference in phosphorylated mTOR at Ser2448 one-hour following unilateral resistance exercise compared to pre-exercise levels (baseline). Furthermore, the addition of vibration to resistance exercise did not alter mTOR phosphorylation. The total mTOR protein content was unchanged.

p70S6K phosphorylation (Figure 3). Resistance exercise led to a significant increase in the levels of phosphorylated p70S6K at Thr389 one-hour following exercise in RT (baseline: 356 ± 131 optical density (OD); post: 5430 ± 1218 OD) and Vbx (baseline: 504 ± 286 OD; post: 5039 ± 2351 OD) ($p<0.05$). The increase in phosphorylated p70S6K was similar between conditions. The total p70S6K protein content was unchanged.

4E-BP1 phosphorylation (Figure 4). One-hour following exercise, levels of phosphorylated 4E-BP1 at Thr37/46 were not significantly influenced by submaximal resistance exercise compared to pre-exercise levels (baseline). The addition of vibration to the exercise did not influence 4E-BP1 phosphorylation. The total 4E-BP1 protein content was unchanged.

Discussion

Whole body vibration applied during resistance exercise has been shown to enhance muscle function (e.g. muscle strength and power) (28, 32, 39-41). In addition, vibration has been associated with reductions in muscular atrophy during long-term bed rest in humans (33, 34, 38) and influences cellular signaling pathways (44). Furthermore, vibration training has been shown to improve the efficiency of a standard fitness training program by returning comparable levels of muscular adaptation while spending less time per exercise session (40 min compared to 90 min) (3).

Resistance exercise acutely increases phosphorylation of mTOR in humans (6-8, 15, 16, 19, 23, 26, 30, 43) and the level of phosphorylation is proportional to the volume of work performed (1, 7, 30). Vibration has been shown to stimulate myogenic processes, possibly through the mTOR pathway, in cell culture experiments (44) as well as improve whole muscle function in humans involved in resistance training combined with vibration (41). We hypothesized that the addition of vibration to a bout of resistance exercise would increase acute levels of mTOR phosphorylation when compared to a single bout of resistance exercise without vibration.

In the current study, we assessed the impact of vibration combined with resistance exercise on activation of the mTOR signaling pathway in human skeletal muscle. The mTOR pathway regulates skeletal muscle growth by influencing protein synthesis. The current subjects performed unilateral leg press exercises with and without vibration followed by tissue sampling to evaluate mTOR pathway activation. We report that resistance exercise combined with vibration does not increase mTOR pathway activation more than resistance exercise alone.

In humans, local vibration of a muscle has been shown to induce alterations at a molecular level. Pietrangelo et al. (36) reported changes in gene expression after local vibration training at high frequency (300Hz) for 12-weeks in elderly subjects who showed signs of sarcopenia in the lower limbs. Local vibration increased the expression of sarcomeric protein genes (actin, destrin, titin, angiomotin). In addition, up-regulation of a splice variant of the PI3K protein (PIK3R3 gene) was measured following local vibration training at a high frequency.

In the current study, mTOR phosphorylation was unchanged one-hour following resistance exercise. Research studies have reported both unaltered (8, 20, 26) and increased (14-16, 19, 23) phosphorylation of mTOR following resistance exercise. Studies showing significant

increases in mTOR phosphorylation appear to involve higher work volumes (load (kg) × repetitions) (14-16, 19, 23). The current subjects performed what was considered to be a typical bout of resistance exercise (4 sets of 10 repetitions). This work volume was smaller compared to previous studies showing a significant increase in mTOR phosphorylation (e.g. 10 sets of 10 repetitions (15, 16)). However, others (8) have reported an absence of change in mTOR phosphorylation at 60 minutes post exercise. In spite of the lack of mTOR phosphorylation they (8) did show increased phosphorylation of mTOR's downstream target, p70S6K, which is consistent with our findings. A possible explanation for the current observations is that the mTOR response may be more transient and/or occur at an early time point with the work volume performed by the current subjects.

Phosphorylation of p70S6K results in the subsequent phosphorylation of the S6 ribosomal protein, a component of the 40S ribosome, that promotes protein translation (2, 4, 6, 25, 30, 42). We observed a significant increase in p70S6K phosphorylation one-hour following the bout of resistance exercise in RT and Vbx. This finding is consistent with previous research examining p70S6K phosphorylation at the one-hour post-exercise time point (8, 16, 19, 23, 26). We observed no difference between exercise conditions.

Considering the similar response between RT and Vbx in p70S6K phosphorylation the current data would suggest that enhanced adaptive responses associated with WBV combined with resistance exercise seen in some studies (31, 33, 34, 38) may not be the result of an enhanced cellular response in the muscle. This conclusion is supported by the results of a 12-month training program in older males who performed exercise training with and without vibration. The study reported that even though both groups increased in muscle mass, there was not a difference between conditions (3). In addition, when comparing resistive exercises with and

without vibration during bed rest, Mulder et al. (35) reported that vibration did not enhance the preventive effect seen with resistive exercises alone. Also, others reporting positive effects of vibration have failed to make comparisons to an exercise only group of subjects (31).

In the active form, 4E-BP1 binds to and inhibits eukaryotic initiation factor 4E (eIF4E). Phosphorylation and inactivation of 4E-BP1 by mTOR disassociates its binding to eIF4E allowing eIF4E to interact with ribosomal proteins that are involved with translation initiation and elongation that result in an increase in protein synthesis (2, 4, 42). It is reported that there is an initial reduction in 4E-BP1 phosphorylation immediately following exercise (12, 14, 15, 23, 30) followed by an increase in phosphorylation as early as 1-hour upon completion of exercise (30).

In the current subjects there was not a significant increase above baseline in 4E-BP1 phosphorylation one-hour following resistance exercise. This agrees with the absence of any change in mTOR phosphorylation. Recent studies have reported similar findings where 4E-BP1 did not increase significantly during the acute hours following resistance exercise yet there were significant increases in muscle protein synthesis (14-16). Based on these findings, Dreyer et al. (16) suggests that 4E-BP1 may not change in response to resistance exercise in humans during the early hours of recovery. The increase in muscle protein synthesis from resistance exercise may be independent of 4E-BP1 (14-17). It has been demonstrated that ingesting essential amino acids independently increases phosphorylation 4E-BP1 and stimulates protein synthesis (22). Ingestion of essential amino acids following resistance exercise significantly increases phosphorylation of 4E-BP1 that was otherwise unchanged following resistance exercise (14, 18). It appears more research is warranted to determine the role of 4E-BP1 phosphorylation following resistance exercise and the ideal time to measure this response in humans.

Whole body vibration research in humans differs in the type and frequency of the vibration being administered (3, 10, 11, 31, 33, 34, 39-41). It is noteworthy that there does not appear to be a universally accepted vibration protocol to administer to human subjects (9). The current study was designed based on vibration parameters (direction and frequency) that have previously produced improvements in muscle function (3, 31, 41) and increases in muscle mass (3, 31). In the current subjects we did not observe any differences in the cellular responses between RT and Vbx.

In cell culture studies, the vibration stimulus (8-10 Hz) administered that showed increased myotube formation through the PI3K pathway in C2C12 cells (44) were lower than the vibration that is typically administered to human subjects (20-50 Hz). Although the vibration parameters of the current study were based on available literature, cellular effects seen *in vitro* by some were not realized *in vivo* in the current subjects. We suggest a possible explanation may be a damping of the vibration stimulus within the body thus reducing the actual vibration experienced in the muscle compared to the vibration generated by the mechanical platform. It has been demonstrated that the vibration stimulus originating at the vibration platform decreases as it travels toward the head, especially at the knee and hip (5, 37). Research has shown that the vibration transmission ratio from the vibration platform to the knee and hip is below 0.56 which demonstrates that the body's natural shock absorbers (meniscus, muscle, bone) dampen the vibration stimulus (5).

Whole body vibration treatment has been shown to increase neural activation of muscle (9, 13, 32, 37). Although researchers have suggested that the increased neuromuscular activity resulting from the addition of vibration to a resistance exercise bout may result in a greater anabolic stimulus in the muscle (35), the current data does not support this supposition at least in

the context of acute responses to resistance exercise. Thus the positive impact that WBV has on muscle function, is indeed, the result of neural factors (9, 32) which would obviously have acute, discreet benefits to performance but lack potential for enhancing chronic adaptations that improve performance.

Augmented cellular responses to direct and local vibration of cells in culture and human muscle, respectively, have been reported in the literature. In the present study, we conclude that phosphorylation within the mTOR signaling pathway is not enhanced following a single bout of submaximal resistance exercise in humans when the resistance exercise is combined with WBV. The time point selection and the intensity of work volume may also have contributed to the findings. The lack of effect in the current subjects may, in part, be the result of the damping effects of the tissues that occurs with the use of WBV platforms. We acknowledge that there are other molecular pathways that may have been influenced that were not measured. However, we would suggest that any whole muscle functional improvements associated with WBV are likely the result of acute neuromuscular effects and not enhanced cellular growth responses.

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Figure Legends

Figure 1. Illustration of experimental set-up used to perform unilateral leg press exercises. The WBV platform (VibePlate 2424, Adrian, MI, USA) was integrated onto a seated leg press foot-plate (Cybex Seated Leg Press, Cybex International, Medway, MA, USA). The position of the subject for performing unilateral leg press exercises is represented.

Figure 2. Representative blot of phosphorylated and total mTOR before (baseline) and one-hour after submaximal resistance exercise (post-ex) with (Vbx) and without (RT) vibration. RT, resistance exercise only; Vbx, resistance exercise with vibration. Data represents phosphorylation of mTOR at Ser2448 at baseline and post-exercise as optical density mean ± SE (n=8).

Figure 3. Representative blot of phosphorylated and total p70S6K before (baseline) and one-hour after submaximal resistance exercise (post-ex) with (Vbx) and without (RT) vibration. RT, resistance exercise only; Vbx, resistance exercise with vibration. Data represents phosphorylation of p70S6K at Thr389 at baseline and post-exercise as optical density mean ± SE (n=8); *Significant increase ($P < 0.05$) vs. baseline (within condition).

Figure 4. Representative blot of phosphorylated and total 4E-BP1 before (baseline) and one-hour after submaximal resistance exercise (post-ex) with (Vbx) and without (RT) vibration. RT, resistance exercise only; Vbx, resistance exercise with vibration. Data represents phosphorylation of 4E-BP1 at Thr37/46 at baseline and post-exercise as optical density mean ± SE (n=8).

Figure 1. Experimental Set-up

Figure 2. Phosphorylated mTOR at Ser2448

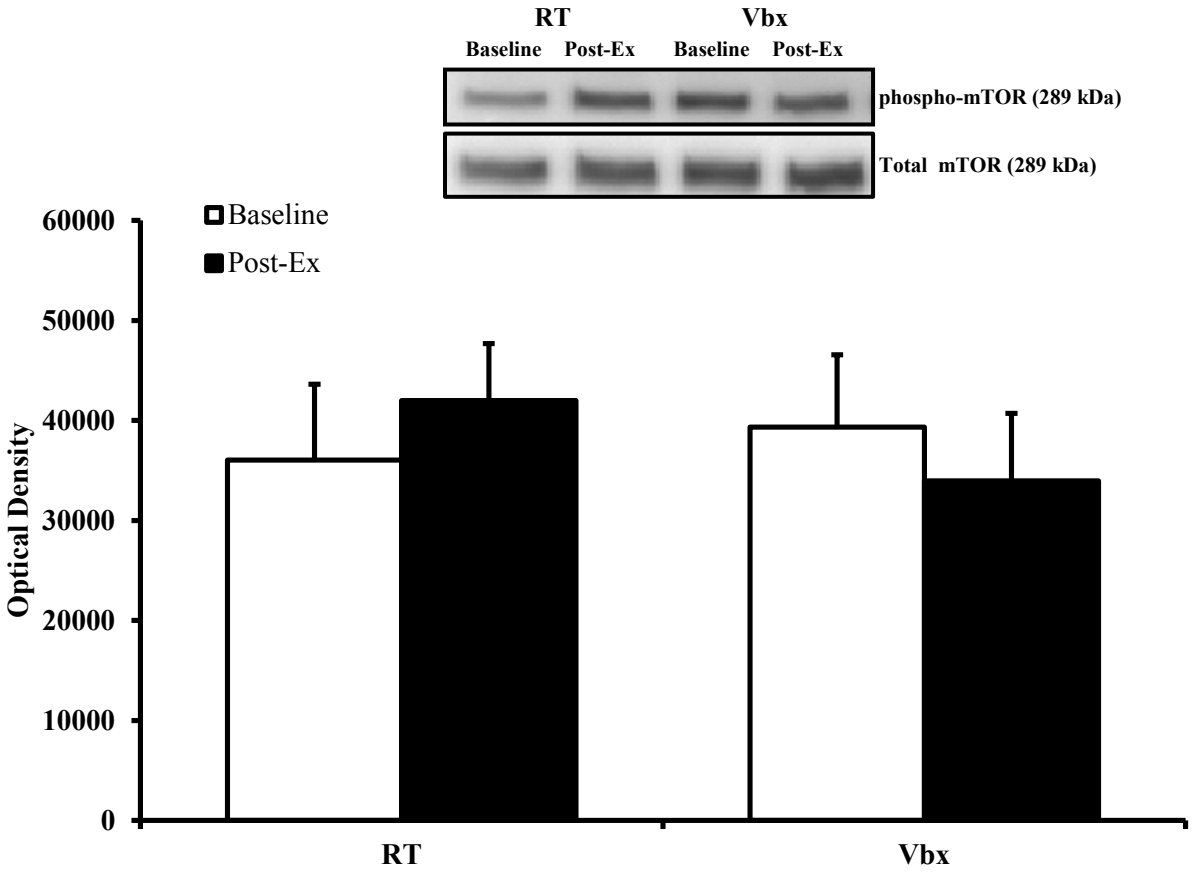


Figure 3. Phosphorylated p70S6K at Thr389

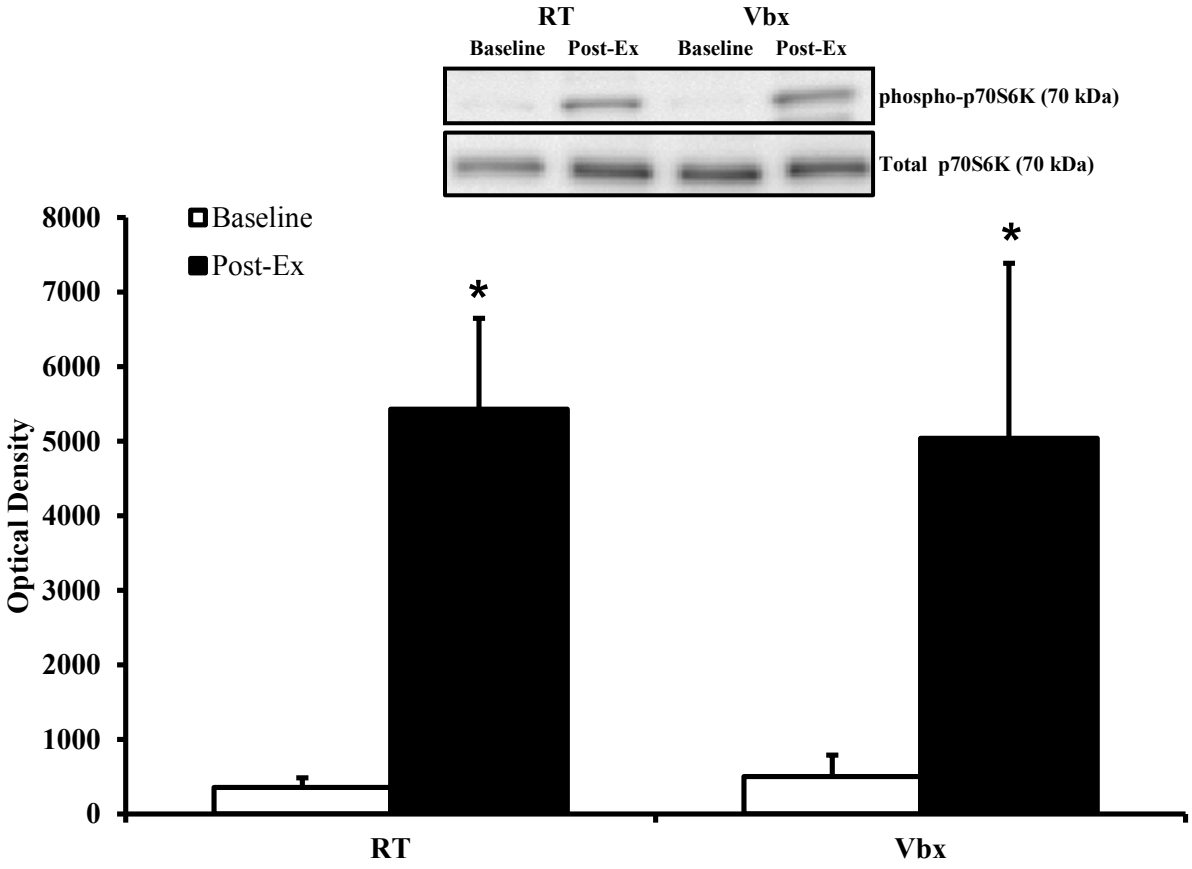
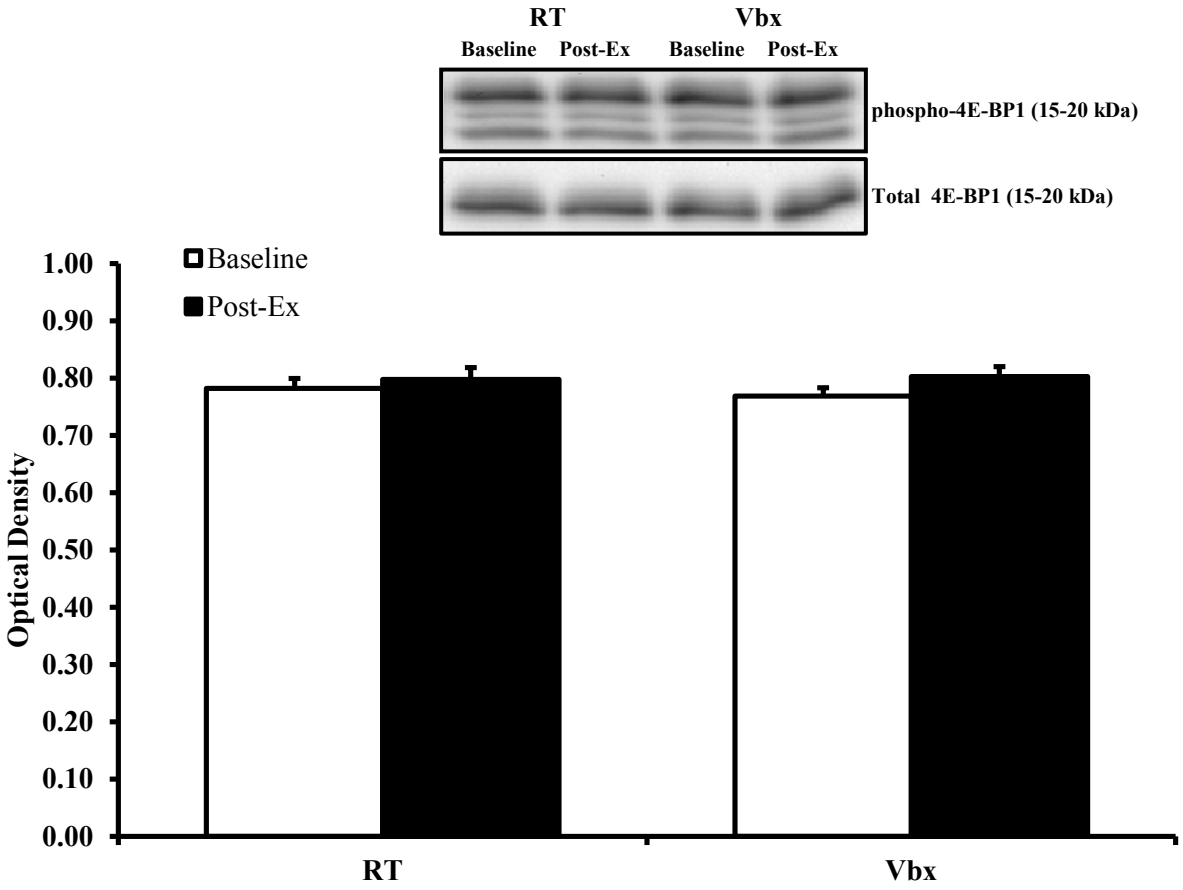


Figure 4. Phosphorylated 4E-BP1 at Thr37/46



Appendix A

Prospectus

Chapter 1

Introduction

Mechanical loading of the muscle is important to stimulate muscular adaptation. The muscle adapts at a cellular level based on the amount of overload which contributes to changes in the whole muscle and functional ability. In recent years, whole body vibration (WBV) has become a method used to mechanically stimulate the muscle. While there are many WBV studies reporting on improvements in muscular strength and power, very few have investigated cellular responses from vibration. There are cellular signaling pathways that respond to stress that are measured to determine the level of response to mechanical overload.

Muscle strength (7, 42, 43, 47, 49, 62-64) and power (20, 22, 43, 49, 62, 64) have been shown to improve when incorporating various vibration treatments with weight training. These positive effects from vibration treatment have been seen with subjects exercising on WBV platforms (7, 20, 22, 47, 62-64), with vibrating cables (42, 43), and with specialized vibration exercise equipment (49). Mileva et al. (49) reported an improved mechanical performance (increased dynamic strength and power) of the rectus femoris (RF) and vastus lateralis (VL) muscles when vibration was applied during knee extension exercise at both low and high intensities. They suggest that superimposed vibration during low-intensity exercise seems to stimulate a response similar to higher intensity exercise. This information is valuable to populations who are limited in the intensity of exercise.

Vibration has been associated with enhanced cellular effect on muscular adaptation in various models (31, 50, 51, 56, 60, 73). During in vitro, vertical vibration stimulated myogenesis in mouse myoblast satellite cells (C2C12) at low frequencies (8 and 10 Hz) (73). Tendon vibration (120 hertz (Hz)) attenuated atrophic process with hindlimb unloading in rats (31) and

WBV (26 Hz) counteracted the atrophic process of long term bed rest in humans (50, 51, 60). Increase expression of phosphoinositide-3-kinase, regulatory subunit, polypeptide (PIK3R3) gene was observed in humans receiving local vibration (300 Hz) (56).

Mammalian target of rapamycin (mTOR) is a key cell signaling pathway involved with muscular growth in humans. The mTOR protein is downstream from phosphoinositide-3-kinase (PI3K) which leads to protein synthesis and skeletal muscle cell growth (72). Wang et al. (73) found that myotube formation with vibration was suppressed with administration of PI3K inhibitor (LY294002). The hypertrophy and myotube formation that occurred with vibration may be contributed to the downstream sensory proteins of PI3K (e.g. mTOR) (73). Given this, we hypothesize that WBV during resistance exercise will augment the activation of the mTOR pathway for muscle growth when compared to resistance exercise only.

Significance

It has been shown that resistance exercise activates the mTOR signaling pathway and increases skeletal muscle growth. If WBV with resistance exercise enhances the mTOR signaling pathway, it will add credibility to the use of WBV as an adjunct tool to resistance training which may result in further benefits from resistance exercise.

Problem Statement

The purpose of this study is to investigate whether WBV with submaximal resistance exercise results in a greater stimulation of the mTOR signaling pathway than submaximal resistance exercise without vibration.

Null Hypothesis

There is no enhanced stimulation of the components of the mTOR signaling pathway with WBV.

Assumptions

1. Subjects will not increase daily activity routine or participate in exercise 24-hours prior to study participation.
2. Subjects will not consume additional food beyond the standardized meal the hours prior to participating in the exercise session.

Delimitations

1. The study will be delimited to a male college age population that is in physically active.

Limitations

Study may be influenced by subjects unable to exercise at provided intensity either because of physical capabilities, mental attributes and/or discomfort from testing equipment (e.g. muscle biopsy).

Terminology

Mammalian target of rapamycin (mTOR) – A sensory protein involved in a cellular signaling pathway that regulates skeletal muscle growth.

p70S6K – 70-kDa ribosomal S6 protein kinase that is a part of the signaling pathway downstream of mTOR.

4E-BP1 – Eukaryotic initiation factor 4E binding protein downstream of mTOR in the signaling pathway.

Atrophy – Decrease in skeletal muscle mass. Results when protein degradation exceeds protein synthesis.

Hypertrophy – Increase in skeletal muscle size (muscle growth). Results when protein synthesis exceeds protein degradation.

One repetition maximum (1RM) – The greatest load able to be lift in a single repetition.

Myogenesis – Formation of muscle tissue.

Whole body vibration (WBV) – Vibrating platform that exposes the entire body to vibration at various frequencies.

Chapter 2

Review of Literature

There is a general understanding that skeletal muscle adapts constantly at a cellular level depending on the physiological demands (1, 34) and muscle activity patterns (35). Whole body vibration (WBV) has been shown to affect muscle function, less has been establish on how WBV affects the muscle at a cellular level that improves performance. This review will begin by addressing the muscular alterations that occur with stress at a cellular level and how these alterations are regulated. With this information in mind, this review will briefly cover the improvements associated by WBV treatment followed by recent cellular findings of vibration stimulus and the next step in understanding what occurs at a cellular level with vibration that may contribute to the improved performance.

Muscular Adaptation

Muscle is affected by activity patterns and the mass of a muscle is determined by the amount of activity it experiences (35). When the muscular activity decreases the muscle will atrophy (decrease in size). An increase in muscular activity may result in an increase in the muscle size, hypertrophy (35). These occur by changes in protein turnover within the skeletal muscle.

Skeletal muscle mass is maintained by a balance between muscle protein synthesis and protein degradation. Hypertrophy occurs when the rate of protein synthesis exceeds the rate of degradation, atrophy occurs when degradation exceeds protein synthesis. A primary way to increase the rate of protein synthesis is to increase the load placed on the muscle, for instance, resistance exercise (5, 9, 75). After a single bout of resistance exercise, muscle protein synthesis increase 2-3 hours post-exercise (24, 25, 54) and can stay elevated up to 24-48 hours (19, 46,

54). The enhanced protein synthesis rate due to the overload placed on the muscle may result in an increase in contractile proteins thus an increase in cross-sectional area (67) which results in a net increase in the size of the muscle fibers and whole muscle (39). Therefore, the size of a muscle is related to the stress it experiences and muscular work is essential for inducing a hypertrophic response (19, 34, 48, 70, 72).

Cell Signaling

Muscular work develops mechanical tension on components of the muscle (75). Contraction activates mechanical-biochemical signaling cascades which alter protein expression by cellular changes in gene expression and other transcription mechanisms in the nucleus (18, 21, 30). The extent of the alteration depends on the intensity, type, and duration of the contraction (2, 75). Cellular adaptations, such as protein synthesis, that follows muscular work are from cumulative effects of transient changes that happen during recovery (57, 75).

Activation of sensory proteins comprises the cascade of events that link the input signal and the cellular functions that occur (72). These sensory proteins compose a variety of signaling pathways, both positive and negative, that are involved in protein turnover (36). The emerging positive sensory protein that regulates muscle growth is the mammalian target of rapamycin (mTOR) (6). The mTOR protein is named by the drug that inhibits its activation, rapamycin (29). Upstream from mTOR is the phosphoinositide-3-kinase (PI3K) and Akt (also known as protein kinase B (PKB)). These proteins comprise the PI3K/Akt/mTOR signaling pathway (mTOR pathway) that leads to protein synthesis and skeletal muscle cell growth (6, 65, 72). Current research provides strong evidence that skeletal muscle growth by increase protein synthesis is mediated by mTOR through the PI3K/mTOR intracellular signaling pathway (4, 6, 9, 14, 26, 29).

Burd et al. (14) results have given confirmation that mTOR and downstream targets are important regulators in protein synthesis in humans.

Mammalian target of rapamycin protein senses input from upstream signals and regulates protein synthesis appropriately. The signals that influence mTOR are insulin, insulin-like growth factor-1 (IGF-1), amino acids (particularly leucine), mechanical tension, and energy turnover. Mechanical loading of the muscle can independently activate the mTOR system. This means that growth factors, such as IGF-1, seem unlikely to be the primary means for inducing muscle growth from muscle loading activities like resistance exercise (72).

Downstream targets of mTOR are important in the involvement in protein synthesis (37). After mTOR has been activated it phosphorylates two downstream targets, 70-kDa ribosomal S6 kinase (p70S6K, also referred to as S6 kinase (S6K)) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1) (4, 6, 9, 25, 29, 55, 69, 73). The phosphorylation and activation of p70S6K phosphorylates the ribosomal S6 protein and other translational components that stimulate protein synthesis (6, 9, 65). Phosphorylation of 4E-BP1 disassociates its binding to eukaryotic initiation factor 4E (eIF4E) allowing eIF4E to interact with ribosomal proteins that are involved with translation initiation and elongation that result in an increase in protein synthesis (6, 9, 65).

To understand the bearing of mTOR in skeletal muscle growth, researchers have inhibited the mTOR protein by administering rapamycin (6, 29). Bodine et al. (6) performed a study on rats to determine the importance of the mTOR pathway in skeletal muscle growth. The study consisted of ablation by bilaterally removing the soleus, medial gastrocnemius and lateral gastrocnemius muscles to produce an overload on the plantaris muscle. The plantaris muscle hypertrophied in rats that did not receive administration of rapamycin. Animals that received rapamycin had a blocked hypertrophic response and activation of downstream targets of mTOR

were blocked. In 2009, Drummond and colleagues (29) showed that mTOR had a key role in stimulating protein synthesis in humans. The study consisted of two resistance exercise groups (control and rapamycin) that performed the same high intense muscle contractions. The human subjects in the rapamycin group received the administration of rapamycin prior to exercise while the control group did not receive any additional treatment. Human subject in the rapamycin group displayed a blocked increase in protein synthesis during the acute hours (1-2 hours) following completion of the exercise session. The control group had an increase of about 40% in protein synthesis following exercise. Additionally, the rapamycin group had a blunted activation of the component of mTOR signaling pathway whereas the control group had an increase in activation. For example, the control group had a 6-fold increase in S6K activation where the rapamycin group was unchanged. Bodine et al. (6) and Drummond et al. (29) studies provide significant support that the mTOR pathways is essential in stimulated muscle protein synthesis following mechanical overload in both rodents and humans, respectively.

mTOR Response

The level of mTOR activation is directly correlated to the loads placed on the muscle, with larger loads stimulating higher levels of activation of the mTOR pathway (4, 14, 29, 44, 69). Studies have reported that the dose of the resistance exercise effects the activation of the mTOR pathway components which is correlated to the protein synthesis response (13, 14, 44). Kumar et al. (44) showed identical changes in expression of the signaling components at intensities 60-90% 1RM when the volume of work load was equalized (%1RM × sets × repetition) which is similar to the typical resistance exercise volume. Researchers have concluded that the signaling and protein synthesis responses may be determined by exercise volume. Most human research investigating the mTOR signaling pathway in response to

resistance exercise involves high work volumes (15, 25, 26, 29, 40, 69). Burd et al. displayed that resistance exercise with low-load high volume stimulates signaling activation and protein synthesis more effective than high-load low volume (14). Lower work volumes do stimulate the signaling response but the effect appears to be more transient and the amplitude is not as great. The magnitude of the signaling response appears to not be affected by gender (26) yet in older humans the response has shown a blunted magnitude (44).

The activation of mTOR and its components gradually increase over the hours after exercise (4, 9). Baar & Esser (4) suggest that components of the mTOR pathway, such as p70S6K, are good markers for changes in muscle mass and may play a role in load-induced skeletal muscle growth (4). The increased activation of the mTOR pathway components occur within one hour post-exercise (10) and remain significantly increased at 3 hours (52) and 6 hours (4, 52) following resistance exercise in animals. In humans, researchers have reported increase in both mTOR and p70S6K as early as 30 minutes after completion of exercise session (15, 69) as well as at 1 hour (15, 25, 26, 29, 40, 44), 2 hours (25, 26, 29) and 4 hours (14) post-exercise. From the mTOR pathway research reviewed in humans, it appears that 1 hour post-exercise seems to be the appropriate time to obtain the muscle sample to evaluate the components of the pathway since this time selection that has displayed consistent activation of the components.

There appears to be a shift in the activation response of the signaling molecules with exercise training (68, 74). Wilkinson and colleagues (74) took untrained human subjects through 10-weeks of resistance training to investigate whether signaling molecules response differently in an untrained state verses a trained state. The study evaluated the signaling response after a single session of resistance exercise in untrained subjects and then following 10-weeks of resistance training. After training, the subjects displayed a greater magnitude of activation of

mTOR and p70S6K than in the untrained state. This increase activation was more rapid in the trained state. The length of the activation response appeared to be shorter following the 10-weeks of training. Researchers suggest that training may increase the efficiency of signaling pathways (68, 74). Therefore, the training status of a muscle displays different responses in the mTOR signaling pathway activation.

Nutrients, primary essential amino acids, can also independently activate the mTOR signaling pathway like resistance exercise (27, 32). Fujita et al. (32) showed that small amounts of ingested amino acids enriched in leucine caused a rapid and large increase in mTOR, p70S6K and 4E-BP1 signaling activation as well as increase stimulation in protein synthesis within 1 hour. Studies have shown an enhance activation of the mTOR pathway after ingestion of amino acids following resistance exercise in humans (24, 28, 33, 40). It appears the incorporating resistance exercise and amino acid consumption may maximize the activation of the mTOR signaling pathway resulting in a large increase in muscle protein synthesis (24, 27, 28). Drummond et al. (27) propose that ingesting essential amino acids shortly after resistance exercise is useful in promoting maximal skeletal muscle growth.

Vibration

Whole body vibration (WBV) has become a popular training tool that has been suggested to enhance muscle performance (e.g. muscle strength and power) with acute bouts of vibration by increasing muscular activation (16, 61). Vibration has been broadly investigated from sport performance to clinical treatment. Russian researchers, Nazarov and Spivak, were the first to use vibration as an exercise intervention to successfully increase muscular strength with trained subjects in the 1980's (42, 43, 45, 64). Whole body vibration has been purported as a beneficial

training tool to increase muscle strength (7, 8, 12, 41-43, 47, 49, 56, 59, 62-64) and power (20, 22, 43, 49, 62, 64) yet few studies have investigated cellular responses (31, 41, 56, 73).

Physiological Effects of Vibration

The intent of WBV is to stimulate skeletal muscles (61). When applied to an active muscle, vibration appears to produce a shift in neuromuscular recruitment patterns by altering the excitation level of the primary (Ia) afferent endings of muscle spindles, which in turn activate the alpha-motorneurons (9, 36, 49, 66). The acute increase in muscle power from WBV may be primarily due to a neurogenic potentiation involving the spinal reflexes and muscle activation (12, 41, 43). There is evidence that WBV increases electromyography (EMG) activity in muscles suggesting that WBV produces greater muscular activation (11, 17, 23). The increase in muscle activation is a result of motor unit recruitment (64).

Muscular activity is determined by the frequency and type of vibration treatment. Rittweger et al. (61) reported a greater EMG mean frequency over the VL after squatting with WBV than without. They suggested that WBV at 26 hertz (Hz) appeared to cause neuromuscular recruitment alterations. Hazell et al. (38) suggests that frequencies of 35 Hz and above (with 4mm amplitude) produced the greatest increase in muscle activity during dynamic squats with vertical WBV. Although, Ronnestad (62, 63) found that 50 Hz stimulated EMG activity to a greater extent than 20 and 35 Hz when compared to traditional resistance training. It has been stated that the body can tolerate vibration frequency from 20-50 Hz when applied through WBV and from 300-500 Hz when applied by local vibration to specific skeletal muscles (56). Tihanyi et al. (71) suggests that the selections of the vibration frequency should depend upon the population and condition of the neuromuscular system. Frequency selection should also consider

how vibration will be administered by either the entire body (WBV) or to a specific muscle/region of muscles (local vibration) (56).

Ronnestad (64) took recreational resistance-trained males through five-weeks of squatting with and without vibration. The squat group with vibration was the only group to increase countermovement jump (CMJ) height. Both groups did increase in 1RM squat and there was a trend that the vibration group increased more in relative strength. Ronnestad (64) suggests that there may be a tendency for vibration to increase maximal strength and power during squat exercise. Although the five-week study did not measure hypertrophy, they suggest that since there was a significant increase in 1RM that there would have been a hypertrophic response to squatting with vibration to a certain degree (64). They support this claim by Necking and colleagues (53) findings that vibration gave a hypertrophic stimulus in rats after the rats displayed an enlargement of muscle fibers (slow and fast twitch).

Studies have theorized that hypertrophy gains are possible; but to date, only two studies have reported muscle hypertrophy gains in humans. The first was a one-year randomized controlled study in men over 60 years of age. They demonstrated that WBV training was as capable in increasing muscle strength (10.9%) and muscle mass (3.4%) as a fitness program in older men with less time per training session (40 min compared to 90 min) though there was no significant difference between groups (7). Researchers stated that the gains in muscle strength and muscle mass contribute to the hypertrophic adaptations along with the neural adaptations that resulted in functional improvements after long-term WBV (7). The other study was a 10-week WBV training in older women (mean age of 76 years) (47). The women subjects increased in muscle strength for the knee and hip extensors. Computed tomography displayed that the cross-sectional area (CSA) of the vastus medialis and biceps fermoris increase 8.7% and 15.5%,

respectively. Researchers suggest the increase in muscle strength contributed to thigh hypertrophy since muscle power was unchanged and there was a lack of change in electromyography activity (47).

Earlier in 1999, Falempin and In-Albon (31) conducted a study to investigate whether tendon vibration could prevent the soleus muscle of rats from atrophying during hindlimb unloading. The rats were mechanically vibrated at 120 Hz on the Achilles tendon for about 3 min/day over a 14-day period of hindlimb unloading. Although tendon vibration did not completely prevent atrophy of the soleus muscle over the 14-days, it did significantly attenuate the loss of muscle mass, the decrease in the fiber CSA (type IIA and IIC), and the decrease in maximal twitch and tetanic tension when compared to the SHAM group. The researchers suggest the duration of the vibration protocol may have been too short to completely prevent the atrophic process of hindlimb unloading. Researchers in human long-term bed rest studies also found that vibration counteracted the atrophic process of bed rest (50, 51, 60). Subjects in these studies underwent horizontal bed rest for 55 (51) or 56 (51, 60) days. The subjects were randomly assigned to either the control or WBV exercise group. Resistive exercises (squats, heel raises, toe raises) with WBV were performed while subjects were still in the bed in a supine position. The WBV platform was attached to the subjects by elastic belts at the hips, shoulders and hands. Moriggi et al. demonstrated that WBV prevented the decrease in CSA (~15%) and torque (~22%) that was associated with subjects in the control group. Researchers also noticed that the vibration treatment prompted changes in muscle fiber type composition and displayed an up-regulation of proteins involved in glycolysis (51). These bed rest studies showed that resistive exercise with WBV appeared to protect muscle size and function (50, 51, 60).

In humans, Pietrangelo et al. (56) demonstrated that vibration induces numerous alterations at a molecular level. The study reported changes in many gene expressions after local vibration training at high frequency (300 Hz) for 12-weeks in elderly subjects showing signs of sarcopenia (an age-related loss of skeletal muscle mass, strength and function) in the lower limbs (56). From the detected changes, phosphoinositide-3-kinase, regulatory subunit, polypeptide 3 (PIK3R3) gene involved with energy metabolism was increased. The PIK3R3 gene is a splice variant of the protein PI3K (3, 58). As discussed earlier, the PI3K protein is a part of the hypertrophy signaling pathway for skeletal muscle (6, 65, 72). Furthermore, local vibration treatment increased the up-regulation of sarcomeric protein genes (actin, destrin, titin, angiomotin) (56) which is an adaptive response from mechanical tension through a series of signaling pathways that increase expression of contractile proteins that will eventually increase in muscular size and strength (26, 29).

Later in 2011, researchers of the Pietrangelo et al. study performed a similar study in young males over 4 months to compare the effects of local vibration to resistance exercise (41). Both studies showed that high frequency local vibration (300 Hz) increased muscular strength and improves neuromuscular performance. Local vibration stimulation significantly improved muscle performance after several weeks of treatment. There were also minor performance improvements after a single session of high local vibration (41). The local vibration group displayed an acute increase in maximal voluntary contraction (MVC) (8.1%) following stimulation which was suggested to be due to improved motor unit synchronization (increased motorneuron recruitment). Over the course of the 4 month training program there was not a significant difference between the local vibration and resistance exercise groups though both

groups increased in MVC. Researchers suggest that high frequency local vibration has beneficial effects that are comparable to resistance exercise training (41).

Myogenesis and Signaling with Vibration

Myogenesis of C2C12 myoblast has been shown to be stimulated by vertical vibration at low frequencies (73). C2C12 is a mouse myoblast satellite cell line that is extensively used in vitro for research in generation and differentiation of skeletal muscle (76). Wang et al. (73) reported a significant increase in the number of myotubes and myotube hypertrophy with vertical vibration at frequencies of 8 to 10 Hz due to the stimulated expression of extracellular matrix proteins (type I collagen and decorin) and myogenic regulatory factors (MyoD and myogenin) in myoblast. They also found that myotube formation was suppressed with administration of PI3K inhibitor (LY294002). The hypertrophy and myotube formation that occurred with vibration may be attributed to the downstream signaling pathways of PI3K (e.g. mTOR) (73).

Conclusion/Rationale

Skeletal muscle modifies its functional capacity in response to a change of stress (16). Vibration treatments have been shown to have physiological effects. It is important to better understand the physiological mechanisms involved in vibration exercise to identify the potential benefits of vibration training. Research has shown that vibration treatment can counteract the atrophic processes (31, 50, 51, 60) and increase in skeletal muscle mass (7, 47). Signaling pathway for skeletal muscle growth have been shown to be involved with the alterations that occur with vibration treatment (56, 73).These alterations provide support to investigate if WBV with resistance exercise enhances the activation of the mTOR pathway when compared to resistance exercise only.

Chapter 3

Methods

Experimental Design

The study will explore the acute effects of a single session of submaximal resistance exercise with and without whole body vibration (WBV) on skeletal muscle hypertrophy signaling through the mTOR pathway. Subjects will perform unilateral leg exercises on a seated leg press (Cybex Seated Leg Press, Cybex International, Medway, MA, USA). The subjects will serve as their own control with one leg performing the exercise concurrent with WBV and the other leg without vibration. The leg that will perform the exercise with vibration will be selected in a randomized and counter-balanced manner. The independent variable is the treatment to each leg and dependent variables are mTOR, p70S6K, and 4E-BP1.

Subjects

Eight, male subjects between the ages of 18-28 years who are not currently engaged in an regular exercise training program will be recruited to participate in this study. To reduce variability each subject will fall within the following anthropometric ranges (height: 66-72 inches, weight: 140-180 lbs., body fat: 10-15%). Subjects will generally be in good health, normally active, and have no recent history of musculoskeletal injury that would limit performance in the exercise protocol. Subjects will be asked to refrain from exceptional physical activity throughout the course of the study and will provide a brief history of their recent normal activity. Human subject approval will be obtained from the university human subjects Institutional Review Board (IRB) prior to beginning the study and all subjects will give their written, informed consent.

Procedures

Individuals interested in participating in the study will be screened for the qualifications outlined above and have their body composition assessed with the Bod Pod® (Life Measurement Instrument, Concord, CA, USA). Those subjects that qualify will be required to read and sign an IRB approved informed consent. Qualified subjects will be introduced to the procedures of the exercise testing and muscle biopsy. Researchers will discuss in detail the procedures of the muscle biopsy by describing the process, what the subject may experience during and after the muscle biopsy as well as the associated risks with this research procedure. The subjects will have the opportunity to familiarize themselves with the equipment (e.g. vibration platform, leg press) and unilateral exercise.

All subjects will be instructed to maintain normal activity levels for at least 48-hours prior to study participation to reduce influencing the baseline mTOR activation level (25, 26, 29). The evening prior to participating in the exercise session, subjects will be fed a standardized meal (60% carbohydrate, 20% fat, and 20% protein, 12 kcal/kg) at 18:00 hours. Each subject will be given a snack (e.g. half a sandwich) to consume at 22:00 hours and will not eat again until completion of the study the following day. The snack will be given to avoid a prolonged fast during the study (25, 26, 29). Subjects will arrive at the Human Performance Research Laboratory at 09:00 hours to be weighed and prepped for muscle biopsy. Researchers will once more describe the exercise and muscle biopsy procedures in detail. Approximately 30 minutes after arrival, the first muscle biopsy will be taken from the vastus lateralis (VL) of each leg. Exercise session will begin with a warm-up 30 minutes following the first biopsy (69). Following the completion of the exercise session, subjects will rest in a comfortable position on the treatment table for one hour and then undergo a second biopsy in each leg. The post-exercise

muscle sample time selection is based on the mTOR pathway literature that has shown consistent results of significant increases in the components of the pathway (15, 25, 26, 29, 40, 44).

Exercise Session

Resistance exercise volume for the warm-up (26, 74) and resistance exercise repetitions (13, 14, 33, 69) are based on previous research that displayed adequate overload to activate mTOR signaling. The exercise load in this study will be determined by the subject's body weight. Our selected percentage of body weight (60%) for the exercise load is based on previous unilateral studies that determined workout load by conducting a 1RM test. The majority of previous studies performed maximal exercise volumes (13, 14, 44, 68, 74). In the present study to prevent masking the possible mTOR activation response from vibration, the percentage of body weight was selected between the maximal and lower volumes of workload/body weight ratios from the previous research (35-45% (14, 74), 70-80% (13, 44, 74), 95-100% body weight (14, 44)). We believe the load selection of 60% of the subject's body weight is sufficient to activate mTOR signaling yet not mask the possible response from vibration. The warm-up and resistance exercise repetitions will be conducted on a seated leg press (Cybex Seated Leg Press, Cybex International, Medway, MA, USA) and the exercising leg will be alternated between sets (e.g. left, right, left, etc.). The first leg to exercise will be counter-balanced between and within treatment.

Subjects will warm-up each leg with one set of 10 repetitions (without vibration) at 30% of their individual resistance exercise repetition load. Three minutes after the warm-up set, subjects will perform four sets of 10RM at 60% of their body weight with a three-minute rest period between sets for each leg. One leg will perform the leg press exercises concurrent with WBV at frequency of 35 Hz (38). The vibration will be administered through a WBV platform

(VibePlate 2424, Adrian, MI, USA) integrated into the leg press foot-plate. Subjects will perform the repetitions at a consistent rate by completing each repetition in approximately 2-3 seconds regardless of treatment. Subjects will have their arms across their chest in an "x" formation and their foot placed in the lower portion of the leg press foot-plate when performing the leg press exercise.

Muscle Biopsies

Muscle biopsy samples will be taken from the lateral portion of the VL of the leg. The biopsy site will be between 15-25 cm from the mid patella (25, 29). The location of the biopsy site will be shaved and disinfected with an antiseptic solution, Betadine. The biopsy site will then be anesthetized with 2 cc of 1% lidocaine (with epinephrine). Five minutes post local anaethesia, a small incision (~1 cm) will be made through the skin and fascia. To perform the biopsies, a 5 mm Bergström biopsy needle will be inserted into the belly of the VL muscle. With suction applied, the muscle sample will be cut followed by withdrawal of the biopsy needle and immediate direct pressure to the incision site with a sterile gauze pad for at least 10 minutes to prevent excess bleeding. The incision will be closed with adhesive wound closure strips and covered with a pressure wrap. The harvested muscle tissue will be immediately blotted, then frozen in liquid nitrogen and stored at -80°C until analysis. The post-exercise tissue samples will be taken from the same insertion point on each leg with the biopsy needle inserted at a different angle so that each biopsy sample will be taken approximately 2 cm apart.

Homogenization

Muscle tissue will be homogenized using chilled homogenization tubes in a 5% homogenization buffer solution (19 µl of buffer to 1mg of tissue) (50 mM Tris-HCl (pH 7.4), 250 mM mannitol, 25 mM NaF, 5 mM Sodium Pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1%

Triton X-100, 50 mM sodium orthovanadate, 1mM DTT, 1 mM benzamidine, 0.1 mM phenylmethane sulfonyl fluoride, 5 µg/ml soybean trypsin inhibitor). Samples will be rotated for 60 minutes then centrifuged at 4°C at 10,000 rpm for 20 minutes. Supernatant will be collected and transferred into fresh tubes and stored at -80°C until further analysis while pellet will be discarded.

Total Protein Analysis

Total protein concentrations will be determined using the BCA micro plate procedure (BCA Protein Assay Kit, Pierce, Rockford, IL, USA). Six dilution levels will be used as protein standards at concentration ranging from 0 to 2000 µg of protein per ml. Protein sample will be combined with the appropriate amount of buffer to obtain a concentrated solution within the range of the standard solution concentrations. Both sample protein and standard solution will be first shaken for 30 minutes then incubated for 30 minutes at 37°C. Following incubation, the absorbance of the sample protein and standard solution will be measured at 560 nm on a micro plate reader using light spectroscopy (VICTOR3, PerkinElmer Life and Analytical Sciences, Shelton, CT, USA). The absorbance will determine the total protein content of the samples by plotting and use of the standard curve fitted by least squares linear regression.

Immunoblotting

Immunoblot analysis will be prepared by combining protein samples of 15 µg with equal volume of loading buffer (0.2% SDS (sodium dodecyl polyacrylamide), 20% Glycerol, 25% 4x buffer, 5% Beta-mercaptoethanol, and 0.0025% bromophenol blue). A molecular ladder (Biotinylated Protein Ladder Detection (#7727), Cell Signaling Technology, Danvars, MA, USA) will be loaded in the first lane. Sample volumes of 10 µl will be loaded into individual lanes of either a 5%, 7.5%, or 10% polyacrylamide mini gel depending on size of protein

(mTOR, p70S6K, 4E-BP1, respectively). The polyacrylamide gel will be submerged in running buffer and run in an electrophoresis unit (Mini Protean II, Bio-Rad Laboratories, Hercules, CA, USA) at a constant 150 V for 60 minutes. After completion of electrophoresis, proteins will be transferred to a polyvinylidene difluoride (PVDF) membrane for 60 minutes at constant current of 100 V. Following protein transfer, the membrane is placed in 5% milk blocking solution overnight at room temperature with gentle rotation. Membrane will be rinsed with PBST solution (1X Phosphate Buffered Saline Tween-20) three times for 10 minutes at room temperature. The membrane will be incubated with the appropriate primary antibody (mTOR, p70S6K, 4E-BP1) diluted in 5% milk blocking solution for 60 minutes at room temperature with rotation. Three 10 minute PBST rinses at room temperature will precede the primary antibody incubation followed by the appropriate secondary antibody incubation for 60 minutes. Three more 10 minute PBST rinses at room temperature will succeed the secondary incubation. Blots will be exposed to the proper chemiluminescent solution (SignalFire™ ECL (#6883), Cell Signaling Technology, Danvars, MA, USA). After one minute of exposure, digital images will be taken and analyzed with Chemi-doc digital imaging software (Biorad).

Antibodies

The primary antibodies used for immunoblotting will be purchased from Cell Signaling Technology (Danvars, MA, USA) (mTOR (Ser2481; 1:1000; #2972), phospho-mTOR (Ser2448; 1:1000; #2971), p70S6K (1:1000; #9202), phospho-p70S6K (Thr389; 1:500; #9205), 4E-BP1 (Ser112; 1:1000; #9644), phospho-4E-BP1 (Thr37/46; 1:1000; #9459)). Anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody will be purchased from Cell Signaling Technology (1:2000) (1:2000; #7074).

Threats to Internal Validity

The threats to internal validity are as follows:

1. We will control the threat of instrumentation by calibrating the instruments prior to every test.
2. The threat of selection bias will be controlled by subjects being recruited on a voluntary base.

Statistical Analysis

A unilateral model was chosen to reduce between-subject variability. We expect this model design to increase the chance of detecting any differences in signaling activation that may occur as a result of WBV treatment rather than between-subject factors. Analysis of covariance (ANCOVA) will be used with the pre-exercise biopsy (baseline) as the covariate to analyze the difference in treatment. The alpha level of $p<0.05$ will be used to indicate statistical significance. Statistical power calculation was based on means and standard deviations from previous research detecting significant changes in mTOR phosphorylation (69). To detect significant difference in the mTOR protein signaling, a sample size of 6 per treatment was determined to be sufficient based on a statistical power of $\beta=0.80$ and an alpha level of $p=0.05$. Sample size will be increased by 25% (total sample size = 8) to account for subject dropout and test failure/error.

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