Molecular effects of alternative hypertrophy methods

Abstract of PhD Thesis

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1. Introduction

The importance of physical activity cannot be overemphasized. The scientific and technical revolution explosion tested the Janus-faced worldview, in which the way of life involved positive and negative changes in people's everyday lives. According to the development of means of transport, people can no longer enjoy their daily life, and therefore the often long walks to work and the positive impact on a test. As a result of this, various recreational trends appeared, which "called" people back to nature, with which the decrease in physical activity was also mitigated. The information revolution has seen telecommunications and information flow in a big way, as communication has become so much faster than people have examined their daily tasks from almost anywhere. This is especially true in today's world. Nowadays, more and more workplaces have switched to the "home office, thus reducing the level of physical activity in society". This is not only an individual problem; in this case, we are talking about a societal problem.

The systematic effects of exercise are supported by an increasing number of popular studies on both healthy and diseased individuals. In addition to increasing physical performance, it also greatly ensures the development and maintenance of good health. The development of the skeletal muscles is a very important factor in all areas of sports, whether it is elite sports or leisure sports. For this development, we also recommend the size of the muscle mass, which can be changed to a large extent with training. The increase in muscle cross-section is one of the most noticeable changes in the effect of training. Muscle mass not only contributes to the aesthetic appearance of the athlete but also to the extent of his exertion. The basis of many sports is strength, which is closely related to the size of the cross-section of the muscle.

The size of the skeletal muscle and its proper condition is significant in terms of performance and can also prevent and control many lifestyle diseases and musculoskeletal problems. After all, skeletal muscle is one of the most massive organs in the human body and thus has a huge influence on the metabolism and hormonal changes of the entire body. As age progresses, muscle mass decreases, which can result in metabolic and endocrinological diseases. In addition, strong and functionally adequate supporting muscles, which can be achieved through regular physical activity, are the basic means of correcting locomotor complaints caused by muscle imbalances and posture errors caused by today's sedentary lifestyle.

The development of muscle cross-section is gaining ground in the world of recreational athletes as well. The world of conditioning rooms is full of recreational athletes whose primary goal is to develop large muscles, rather than regular exercise out of a desire for a healthy lifestyle. The developed musculature, which lends an aesthetic appearance, has also been significant throughout our history. Just think that the great warriors and conquerors of history were all depicted with huge muscle mass by the artists of certain cultures, not to mention the athletes of the ancient Olympic games and the characters of mythologies. In addition to all this, looking at it from an evolutionary point of view, the large muscle mass led to the conclusion of a higher testosterone level, which was typical for alpha males within the given species.

Researchers have long been concerned with the physiological changes that occur during training, including hypertrophy and its effects on multiple segments of life. The molecular pathways and signaling systems of hypertrophy are not a new field of research in the sports and life sciences, but there are still unsolved areas to this day. In the course of the research, the tests did not only focus on classical resistance training but different methods were used to achieve an increase in muscle cross-section.

In this thesis, I will explain two types of hypertrophy methods, which are also researched, and we refer to as alternative hypertrophy methods. One of these methods is the so-called "compensatory hypertrophy" model or the "overload-induced hypertrophy" model used in English, which is a procedure performed on rats or mice. With surgical intervention, the synergists of certain muscles of the hind leg are removed, with which a "compensatory" adaptation can be achieved in the remaining muscle. This procedure is used to achieve rapid and large-scale hypertrophy and forms the methodological basis of many pieces of research.

The other alternative method is occlusion or vasoconstriction training, native to Japan, which has become a popular training method in Japan and since then in other parts of the world. This type of alternative training method can appear in all areas of sports, but it also occupies a popular place in rehabilitation. In both cases, I used the term alternative, referring to the fact that we are not trying to achieve muscle cross-sectional growth with training methods in the ordinary sense.

The compensatory hypertrophy model is not discussed as a training method. During the model, the animal is subjected to continuous loading, as the existing plantarflexor muscle is responsible for maintaining body weight, which is continuously overloaded during the 2-week treatment. In the previous research of our laboratory, we discovered the effect of this method on activating

the SIRT1 protein, which revealed a completely new direction of the molecular signaling processes of hypertrophy.

Occlusion training is a kind of combination of different training with limited blood supply to the limbs. This type of load can be classic resistance training or even endurance training. In my thesis, I will also cover the health risk factors of occlusal load and its positive physiological effects. This method is used during the load while performing the exercises. During our research, we did not apply this method based on classical occlusion procedures but instead presented a novel use of this training method. The occlusion compression we used was performed during the rest period of the training, the rationale and effects of which will be covered in detail.

In this thesis, I discuss the role of different anabolic and catabolic pathways and their changes as a result of different methods. In addition, I also describe mitochondrial dynamics and the redox state and its changes.

In order to learn about the role of the silent mating type information regulation 2 homolog 1 (SIRT1) protein in hypertrophy, some research were carried out during the past decade, which form the basis of my thesis. Considering the results of these researches further, the topic of my thesis was formed, through the results of which we get a more complex picture of the interactions of the proteins involved in hypertrophy and their pathways.

The novelty of the research that forms the basis of my thesis lies in the fact that during the tests conducted with the compensatory hypertrophy model, we present novelties in the exploration of the various molecular pathways and their relationships, while by applying a previously unexplored method of occlusion training, we got a more complete picture of its physiological effects.

Sports science, as an interdisciplinary science, provides many opportunities for the birth of new research. My choice of topic fell on hypertrophy and its alternative possibilities, since I have been interested in the possibility of increasing the muscle cross-section since the beginning of my university education. The physiology of classical hypertrophy training has been a researched field for a long time, and for this reason, in my thesis, we aimed to further learn about non-classical hypertrophy methods.

2. Objectives

Our main objective is the further detection of the physiological effects caused by hypertrophy, which not only covered protein synthesis and degradation, but also the detection of changes affecting the energetic state of the muscle.

We defined our studies in 2 main directions: human and animal model research. Our research covered alternative hypertrophy tests, one of which is the compensatory hypertrophy model and the other is occlusion training.

We formulated our hypotheses as follows.

1. In the animal model study, we assumed that:

1.1. As a result of the application of the compensatory hypertrophy model, markers of protein synthesis increase and markers of the catabolic pathway decrease in the compensation group compared to the control group.

1.2. Mitochondrial biogenesis markers increase and mitophagy markers decrease in the compensation group compared to the control group as a result of the treatment.

1.3. As a result of the treatment, the level of antioxidant markers increases in the compensation group compared to the control group.

2. For the human study, we assumed that:

2.1. After acute resistance training combined with occlusion loading during the rest period between loads, the levels of muscle anabolic markers increase and catabolic markers decrease in the occluded leg compared to the control leg.

2.2. Mitochondrial biogenesis and capillarization markers are elevated in the occluded leg compared to the control leg.

2.3. The level of antioxidants increases with exercise in the occluded leg compared to the control leg.

3. Material and method

3.1. Overload-induced model

3.1.1 Animals

Eighteen middle aged (8 months) male Wistar rats were randomly divided into a control (C) and a hypertrophied (H) group. Animals were held in a thermoneutral room on a 12:12 h photoperiod and were provided with food and water ad libitum. The entire experiment was carried out in the Research Center for Molecular Exercise Science, University of Physical Education of Hungary and approved by the National Ethical Committee (63/2/2017 and PE/EA/62-2/2021).

3.1.2. Synergist Muscle Ablation

The main synergist muscles (gastrocnemius, soleus) of the plantaris muscle were surgically removed. All the operations were carried out under deep anaesthetic conditions with pentobarbital sodium (50 mg/kg). The surgical procedures were performed bilaterally as described previously [8]. The neural and vascular supplies of the plantaris muscle remained intact. The control group underwent a sham operation when the tendon of the plantaris and its synergist's tendon were separated carefully but the soleus and the gastrocnemius muscles were not damaged or removed. After the operation and for the next two days the animals were administered analgeticum. The overload period lasted for 14 days and the animals were monitored for the whole period. On day 14 the food was taken away and the next morning the animals were euthanized (decapitation) after an overnight fast. The plantaris muscles were collected immediately after the removal of the fat and the connective tissues. The muscles were weighted and frozen in liquid nitrogen and stored at −80 °C until further analysis.

3.1.3. NAD^+ Measurement

A NAD^+/NADH Assay Kit (ab176723) was used to measure the NAD^+ levels in the plantaris muscles according to the manufacturer's instructions. Plantaris muscles were homogenized in NADH/NAD^+Lysis buffer. Then the samples were centrifuged and separated into treated and untreated parts. The samples and 25 µL diluted NADH standards were loaded into 96-well microplates in duplicate. Then, 25 µL of NAHD/NADH Control Solution was added to the standards and 25 µL of NADH Extraction Solution or NAD^+Extraction Solution were added, respectively, to the NADH and the NAD^+samples. After this the plates were heated at 37 °C for

15 min for NAD^+/NADH decomposition. Then 25 µL of NAD^+/NADH Control Solution were added to the standards and 25 µL of NAD^+Extraction Solution or NADH Extraction Solution were added to the NADH and NAD^+samples, respectively. Then, 75 µL of Reaction Mix were added to all wells. For 2 h the optical density was measured every five min at ex485 and em538 nm wavelength.

3.1.4. Western Blots

The muscle homogenates were procreated by Ultra Turrax homogenizer (IKA, Staufen im Breisgau, Germany) with lysis puffer. The samples were electrophoresed on 6–15% polyacrylamide (SDS-PAGE) gels. The proteins in the samples were transferred into PVDF membranes. Then, the membranes were blocked with BSA (0.5–5%) or Milk (5%) for 2 h at 4 °C. After blocking the membrane were incubated with primary antibody at 4 °C overnight. Antibody list: SIRT1 1:1000 (Ab:110,304), S6 1:5000 (Cs:2217S), pS6 1:5000 (Cs:5364S), AKT 1:3000 (Cs:46915), mTOR 1:1000 (Cs:29835), p-mTOR 1:1500 (Cs:5536), FOXO1 1:1000 (Cs: 9454), Sestrin2 1:5000, (Ab 23602), AMPK 1:1000 (Cs: 2532), p-AMPK 1:1500 (Cs:2535), NAMPT 1:500 (Ab45,890), Cytochrome C 1:1000 (Sc-7159), COX4 1:2500 (Sc-69,359), Sirt3 1:10,000 (Proteintech:10,099-1-AP), NRF2 1:1000 (Ab:31,163), SOD2 1:3000 (Invitrogen:PA5-80048), PINK1 1:1000 (Affinity: DF7742), OGG1 1:1000 (Proteintech: 15125-1AP), GAPDH 1:3000 (Sigma: G8795), Pax7 (1:500, sc-81648; Santa Cruz, Dallas, TX, USA), NRF1 (1:1000, sc-33771, Santa Cruz), PGC-1α (1:3000, KP9803; Calbiochem, San Diego, CA, USA), and α-tubulin (1:5000, T6199; Sigma-Aldrich, St. Louis, MO, USA). The next day the membranes were washed three-times with Tris-buffered saline-Tween-20 (TBST) at room temperature and incubated with HRP-conjugated secondary antibody for 2 h at 4 °C. After that the membranes were washed again with TBST three times at room temperature. Then the membranes were incubated with chemiluminescent substrate and protein bands were visualized on X-ray films. The bands were quantified by ImageJ software. The relative density was calculated to our housekeeping proteins GAPDH and α-tubulin.

3.1.5. Measurement of H2S with the Monobromobimane Method

H2S assay was based on a previously published method adapted here for tissue lysates. First, approximately ~10–20 mg of the tissue samples were disrupted by a dismembrator. Alkylation/lysis was carried out by the addition of 500 µL PBS set to pH 8.0 containing 1 mM monobromobimane (Sigma Aldrich, St. Louis, MO, USA) in a light-protected environment. After a short sonication on ice the solutions were incubated for one hour at 37 °C in the dark. The reaction was stopped by the addition of 50 µL 50% TCA followed by centrifugation at

12,000× g 4 °C for 10 min to remove precipitated proteins. Supernatants were removed and transferred into HPLC vials for measurement, and the remaining pellets were redissolved in 300 µL 4% SDS/0.1 M NaOH for BCA protein assay. Bimane labeled species from the supernatants using 3 µL injection volumes were separated on a Phenomenex Luna C18(2) 250 × 2.0 mm × 3µm column on a Thermo Ultimate 3000 UHPLC system (Thermo Fisher, Waltham, MA USA). A linear gradient elution using solvents 0.1% TFA/H2O (A) and 0.1% TFA/ACN (B) was carried. The fluorescent detector was set to excite at 390 nm and detect emission at 475 nm. Quantitation was conducted by establishing a calibration curve by derivatizing standardized H2S solutions.

3.1.6. Measurement of CBS Activity

Frozen tissue samples of ~10–20 mg were disrupted by a dismembrator (B. Braun 853162) followed by the addition of the lysis buffer (150 mM KCl, 50 mM HEPES pH 7.4, 0.1% CHAPS, 2% protease inhibitor cocktail) of 400 µL. After a brief sonication on ice, tubes were placed on a rotator for 30 min at 4 °C. After centrifugation at 12,000× *g*, 4 °C for ten minutes, supernatant protein content was measured by BCA assay. All samples were diluted to 1 mg/mL protein concentration using the lysis buffer. The prepared solutions were used to carry out the CBS activity assay exactly as described previously. In brief, samples were mixed with cofactors (SAM, PLP) and substrates homocysteine (prepared fresh from HCys-thiolactone) and stable isotope labeled serine (2,3,3-D-serine, Cambridge Isotope Laboratories, Inc., Tewksbury, MA, USA) followed by four hours of incubation at 37 °C. Reaction mixtures were quenched using with ”Reagent 1” of the EZ:faast kit (Phenomenex, Torrance, CA, USA) spiked with a known amount of stable isotope labeled cystathionine (3,3,4,4-D-cystathionine, Cambridge Isotope Laboratories, Inc.) as an internal standard. Sample preparation and measurement with the EZ:faast kit was carried out following the manufacturers manual. For the HPLC-MS/MS measurements a Thermo Vanquish (Thermo Scientific, USA) UHPLC coupled to a Themo Q Exactive Focus MS was used and the SRM transitions of 4813 → 421 (product) and 4833 → 423 (internal standard) were monitored. Specific activities were calculated from the amounts of cystathionine produced and the protein contents of the samples.

3.2. Blood flow restriction study

3.2.1. Blood flow measurement

The occlusion pressure was approximately 230 mmHg using the standard cuff. Doppler 2-dimensional real-time ultrasound examination (General Electric, Boston, MA, USA) was used to detect blood flow in the restricted leg during the rest periods. The results from our pilot study previously demonstrated that this pressure was well-tolerated by the participants and also suggested that greater levels of restriction during the resting periods could enhance the effects of BFR.

Blood flow velocity was measured before, during, and immediately after depressurizing the cuff, using 7.5–10.0 MHz frequency, with a linear transducer placed at the popliteal artery (at 60°), by an experienced physician. The blood flow was detected by Doppler before starting the exercise, after every 2 sets of squats, after the last set, and 5 min after the last exercise bout. Resting diastolic diameter (mm) was averaged over 30 cardiac cycles. Blood flow (mL/min) was calculated as (time-average mean velocity × $\pi r2$) × 60, where r is the radius of the artery lumen. Resting blood flow was averaged over 20 cardiac cycles. Synchronized diameter and velocity data enabled calculation of blood flow.

3.2.2. Acute exercise protocol

The 1st participant started the exercise protocol at 8:20 a.m. Participants were instructed to avoid food consumption 10 h prior to the exercise protocol. The maximal load of squatting was measured as 1 repetition maximum (RM). The 1RM measurements were conducted 1 week prior to the occlusion protocol. All participants were familiar with the squatting exercise. Briefly, after a 10-min warm-up on a cycling ergometer, participants performed 10 repetitions at a load one-half their body weight and 4–6 repetitions at total body weight. In the testing phase, load was gradually increased to reach 1RM by following the National Strength and Conditioning Association (USA) guidelines. In 4 trials, all participants reached 1RM. Between sets there was a 2-min rest period.

All participants performed the squat exercises at 70% 1RM in 7 sets with 10 repetitions. Between sets, during the 2-min rest period, right leg BFR was performed using the Mizuho BFR system (Tourniquet #8; Mizuho, Tokyo, Japan) with an 11-cm-wide cuff. The left leg served as a control. During the 2-min rest periods between sets, immediately after the last squat,

BFR was applied with repeated pumping, which increased the pressure of the cuff to 230 mmHg for 1 min. The cuff was then removed. Between each set, subjective discomfort feelings were recorded using the conventional 20-point Borg scale.

3.2.3. Muscle biopsy samples

To determine the expression level of the corresponding mRNA, micro biopsy samples were taken for miR and protein fractions using a semiautomatic needle (EASY-RAM 14G (gauge) × 100 mm (length); RI.MOS., Mirandola, MO, Italy). Local anesthetic was applied (20 mg/mL lidocaine-hydrochloride; EGIS, Budapest, Hungary) to the vastus lateralis muscle of both legs 2 h after the last exercise set. The biopsy resulted in about 10 mg of muscle samples. The samples were divided in half, frozen in liquid nitrogen, and kept at –80°C for subsequent RNA and protein extraction procedures.

3.2.4. RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR) of the mRNA and miR transcripts

Expression levels of target genes were measured by qRT-PCR. The cycle threshold (Ct) values of each PCR run were recorded. The housekeeping gene was selected by using the RefFinder online tool, which uses the common methods for GeNorm, Normfinder, BestKeeper, and the comparative ΔCt method for identifying housekeeping genes. Comprehensive gene stability (gene geomean of ranking values) was calculated. Of the 5 candidate genes, the 28S RNA showed the best expression stability (mean = 20 Ct, SD = 0.38 Ct, coefficient of variation = 1.9).RNA extraction (NucleoSpin RNA mini; Macherey-Nagel, Düren, Germany) and cDNA synthesis (SensiFAST™ cDNA Synthesis Kit; Bioline, London, UK) were performed according to the manufacturers' instructions. Specific gene products were amplified by qRT-PCR using primer pairs. The following applied primers were also used: paired box 7 (Pax7), Pax3, mTOR, silent mating type information regulation 2 homolog 1, nuclear respiratory factor 1 (NRF1), vascular endothelial growth factor (VEGF), insulin-like growth factor 1, peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α), forkhead box protein O1, 28S RNA, mitochondrial transcription factor A, hypoxia-inducible factor 1α, lupus Ku autoantigen protein p70 (Ku70), RAC-β serine/threonine-protein kinase 1(Akt1), Akt2, superoxide dismutase 1, superoxide dismutase 2, and U6 small nuclear RNA (U6).

The most stable gene expression was found to be 28S RNA. Consequently, this gene expression was used as the reference gene (BestKeeper). The mRNA samples were annualized using the SYBR Green real-time system (Thermo Fisher Scientific Inc., Waltham, MA, USA).

The qPCRs for each miRNA (10 μL total volume) were performed in triplicate, and each 10-μL reaction mixture included 2.4 μL of 10 × diluted reverse transcriptase product. Reactions were run on a PRISM 7900HT Fast Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Twofold dilution series were performed for all target miRNAs to verify the linearity of the assay. To account for possible differences in the amount of starting RNA, all samples were normalized to U6, which proved to be the most stable (mean = 23.1 Ct, SD = 0.44 Ct, coefficient of variation = 1.88). All reactions were run singularly and quantified using the Ct (ΔΔCt) method.

3.3. Statistical analysis

The Statistica 13 program was used for the statistical analyses. The results were subjected to the Shapiro-Wilk normality test. If the samples showed a normal distribution, a 2-sample t-test was used. If the distribution was not normal, the Mann-Whitney U test was used. To determine variance analysis, we used peig Repeated ANOVA with Tukey HSD post hoc analysis. We used Pearson's correlation to establish the relationship between the variables. The significance level was defined as $p<0.05.5$.

4. Results

4.1.1 Anthropometric changes

After 2 weeks of treatment, no significant difference was found between the O and C groups in the body weight of the animals (C=399±17g; O=381±34.12g). We found a large increase in the weight of the plantaris muscle (C=0.383±0.01g; O=0.486±0.05g) in the O group compared to the C group as a result of the treatment ($p<0.01$). In the plantar weight/body weight % ratios (C=9.6%±0.005; O=12.7%±0.01) we also discovered a significant difference in the O group compared to the C group ($p<0.01$). From these results, it can be seen that the plantaris muscle underwent a large change as a result of the procedure.

4.1.2. Biochemical changes measured in the plantaris muscle

As a result of the treatment, the Akt protein level showed a significant increase in the plantar muscle of the animals in group O compared to group C ($p<0.01$). The mTOR protein level also increased significantly in group O compared to group C ($p<0.05$). The phosphorylation state of the mTOR protein also increased significantly in group O compared to group C ($p<0.05$). The level of ribosomal S6 protein was significantly increased in group O compared to group C ($p<0.01$). The level of ribosomal protein S6 phosphorylation was also increased in group O compared to group C ($p<0.01$). The SIRT1 protein level significantly increased as a result of the treatment in group O compared to group C ($p<0.01$). We detected memory in all markers of the anabolic pathway that we investigated as a result of the 2-week procedure. NAMPT protein showed an elevated level in the study group compared to group C ($p<0.05$).

The ratio of phosphorylated mTOR to total mTOR protein showed no significant difference between the two groups. The levels of ribosomal S6 phosphorylation and total S6 protein status were also not increased in the O group compared to the C group.

The level of FOXO1 protein significantly decreased in group O compared to group C as a result of the treatment ($p<0.01$). The level of Sestrin2 protein showed a significant decrease in group O compared to group C ($p<0.01$). The level of AMPK protein significantly decreased in group O compared to group C as a result of the treatment ($p<0.01$). The level of the phosphorylated state of the AMPK protein did not show a significant difference between the two groups as a result of the treatment.

Cytochrome C protein level showed a significant decrease in the O group compared to the C group as a result of the treatment ($p<0.05$). The COX4 protein level showed a significant

decrease in the O group compared to the C group as a result of the treatment ($p<0.05$). The level of NRF2 protein showed no significant difference between the two groups as a result of the treatment. The SOD2 protein level showed no significant difference between the two groups as a result of the treatment.

The protein level of SIRT3 showed a significant decrease in the O group compared to the C group as a result of the treatment ($p<0.01$). The PINK1 protein level did not change significantly between the two groups as a result of the treatment.

The NAD^+/NADH ratio shows the energy and redox state of the cell. We found no difference in the amount of NADH between the two groups as a result of the treatment. The amount of NAD^+ decreased significantly in the O group compared to the C group as a result of the treatment ($p<0.05$). The level of NAMPT protein, as a precursor involved in the biosynthesis of NAD^+, was significantly increased in group O compared to group C ($p<0.05$).

The level of the OGG1 protein, which allows us to infer the DNA damage and the redox state of the cell, significantly decreased as a result of the treatment in group O compared to group C ($p<0.05$). Ac p53 protein, which shows the redox state of the cell, showed no difference between the two groups as a result of the treatment.

CBS is a key enzyme involved in H2S synthesis. As a result of the 2-week treatment, the amount of the CBS enzyme did not show a significant change between the two groups. The amount of H2S did not show a significant difference between the two groups as a result of the treatment.

4.2. Changes occurring as a result of human occlusion training

4.2.1. Results of blood flow parameters

The velocity of the systolic and diastolic blood flow was examined using a Doppler ultrasound imager. The systolic blood flow velocity increased significantly at time 4 and 5 compared to the value measured at time 3 ($p<0.05$) and showed a significant decrease at time 6 compared to the value measured at time 5 ($p<0.05$).

Diastolic blood flow showed a significant increase at time 6 compared to the value measured at time 5 ($p<0.05$). Based on the results of the representative Doppler images, we can say that the values measured at 3 times (at rest, peak flow and 5 minutes after exercise) showed increasing blood flow velocity and decreasing peripheral resistance.

4.2.2. Biochemical changes occurring as a result of occlusion training

A significant increase was found in the levels of Akt2 ($p<0.05$), NRF-1 ($p<0.05$), VEGF ($p<0.05$), Pax7 ($p<0.01$) and Ku70 ($p<0.05$).

2 hours after exercise, we found a significant decrease in the level of miR-206 in the test leg compared to the control leg ($p<0.05$).

The change in the Pax7 gene mRNA level and the miR-206 miRNA level showed a correlation ($r2 = 0.33$, $r = 0.577$, $p = 0.031$).

During the biopsy sampling, due to the small amount of extracted muscle tissue, we were able to perform few tests. Therefore, as the next step of our research, we selected 3 proteins involved in different physiological processes, the proteins Pax7, NRF1 and PGC1A, which were examined by Western blotting. There was no significant change in the level of Pax7 protein in the occluded leg compared to the control leg. There was no significant difference in the level of NRF1 protein between the two legs as a result of training. We also found no significant difference in the level of PGC1A protein in the occluded leg compared to the control leg as a result of exercise.

5. Conclusions

The importance of skeletal muscle hypertrophy cannot be questioned, since, if we examine its rich literature, we quickly realize that it is an extremely important research area not only in sports science, but also in other areas of life sciences. The goal of our research was to gain a deeper understanding of the physiology of alternative hypertrophy methods. The 2 directions of our studies, which can be interpreted as a whole as a complement to each other, brought us closer to expanding the wide range of knowledge of hypertrophy. Nevertheless, we will discuss the consequences of the changes caused by the two methods separately.

The animal model we used resulted in a large degree of hypertrophy in the treated group. The explanation for this can be found both in the increase in the level of proteins involved in the anabolic pathway and in the decrease in the amount of proteins in the catabolic pathway. The large increase in the Akt protein, which is the cornerstone of the AKT-mTOR molecular signaling system, resulted not only in the further activation of the aforementioned pathway, but also in the inhibition of catabolic pathways through the reduction of Sestrin2, which activates the FOXO1 protein. By phosphorylating the mTOR protein, Akt not only increased the role of mTOR in protein synthesis, but also directly inhibited AMPK and the anti-anabolic and catabolic proteins FOXO1. From these results, we can conclude that due to the activation and inhibition of the above-mentioned processes due to the large and continuous mechanical load, protein synthesis, i.e. hypertrophy, increased, which was supported by the increased weight of the plantaris muscle and the total and phosphorylated version of the S6 ribosomal protein.

In recent times, research has increasingly focused on the role played by the SIRT1 protein in hypertrophy, in addition to its already wide-ranging physiological effects. In our research, the increased amount of SIRT1 protein was associated with increased protein synthesis and an increase in its markers, which is clearly shown by the direct effect on the reduced level of the catabolic protein FOXO1. Accordingly, according to: 1.1. As a result of the application of the compensatory hypertrophy model, markers of protein synthesis increase and markers of the catabolic pathway decrease in the compensation group compared to the control group.

WE ACCEPT

In addition to the SIRT1 protein, NAMPT, which is the precursor of NAD^+ biosynthesis, also showed an increase, which supports the continuous demand for NAD^+ and thus the SIRT1 activation as a result of the treatment. The gas transmitter H2S, which has a SIRT1 activation

effect, was administered externally and did not increase in the study group as a result of the treatment. From this, we conclude the treatment-dependent activation of the SIRT1 protein.

The reduction of mitochondrial proteins such as COX4 and Cytochrome C would at first suggest a decrease in the number of mitochondria, but the lack of statistical difference detected in the mitophagic protein PINK1 and SIRT3 allows us to conclude that the amount of mitochondria in the muscle did not decrease. There was simply no increase in the amount of existing mitochondria, which may be related to the massive hypertrophy and the already fast glycolytic type of muscle. Based on these, our hypothesis that: 1.2. mitochondrial biogenesis markers increase and mitophagy markers decrease in the compensation group compared to the control group as a result of the treatment. WE DO NOT ACCEPT, as the mitochondrial markers decreased, while the mitophagy marker showed a stagnant state.

Antioxidants that protect against oxidative damage are known to have higher levels in slow-twitch than fast-twitch fibers. This is presumably due to the increased number of mitochondria and the resulting higher amount of free radicals. In the plantaris muscle, which is a fast fiber type muscle, we found lower NAD^+ activity, OGG1 and Sestrin2 levels as a result of the treatment. All three molecules allow us to infer the redox potential of the cell, from which we can draw the conclusion that a reduced environmental state existed in the muscle as a result of the model. Consequently, 1.3. our hypothesis: as a result of the treatment, the level of antioxidant markers increases in the compensation group compared to the control group, WE DO NOT ACCEPT it, as Sestrin2, SIRT3, OGG1, NAD^+ showed a decrease.

In the second half of the 20th century, occlusal loading was considered a common training method in various parts of the world. In this study, we combined classical resistance training with occlusion treatment. Our test results revealed that mRNA transcription of certain genes increased in the treated leg as a result of the acute load. The transcription of genes involved in protein synthesis and thus hypertrophy processes, such as AKT2, Pax3, Pax7 and KU70 genes, increased already two hours after the exercise. From these results, we can conclude that both satellite-cell-dependent and satellite-cell-independent pathways of protein synthesis were activated.

The increase in NO level caused by compression can result in satellite cell activation, which was also demonstrated in our present study. Despite this, the level of the Pax7 protein did not increase as a result of the treatment, which can be assumed from the short time between the load and the biopsy. Based on hypothesis testing: 2.1. After acute resistance training combined

with occlusion load, the level of muscle anabolic markers increases and catabolic markers decrease in the occluded leg compared to the control leg. We partially ACCEPT, as the level of the anabolic markers AKT2, Pax7, Pax3, KU70 increased, but this cannot be said for the other anabolic markers examined. The expression of the FOXO1 gene did not show a decrease as a result of the treatment.

The expression of the VEGF gene also showed an increase as a result of the treatment, with which the body wants to compensate for the lack of oxygen from the large amount of compression by increasing the capillary network. In addition to the elevated VEGF and NRF1 levels, we did not find an increase in the PGC1A mRNA level, considered as the master regulator of mitochondrial biogenesis. According to our assumption, this anaerobic energy demand resulting from a high-intensity and oxygen-deficient state did not change as a result of the treatment. According to our next hypothesis: 2.2. Mitochondrial biogenesis and capillarization markers are elevated in the occluded leg compared to the control leg. We partially ACCEPT, as VEGF as a capillarization marker showed an increase, while there was no significant difference in the mitochondrial biogenesis markers as a result of the treatment.

Transcription of the NRF1 gene, which has an antioxidant effect, also increased, which suggests the initiation of protection against free radicals produced by high intensity. On the basis of which our hypothesis: 2.3. Antioxidant levels increase with exercise in the occluded leg compared to the control leg. We partially ACCEPT, as we only found a difference in the mRNA marker.

Perhaps the most surprising point of our results is the decrease in the reduced miR-206 miRNA and its correlation with the increased Pax7 level. This microRNA plays a role in the inhibition of anabolic processes, which was clearly inhibited by the occlusion treatment in our present research. We could not reproduce this result with our chronic occlusion treatment, however, miR-1 and miR133a showed a significant decrease as a result of the treatment (Torma et al. 2021).

For this thesis, we chose the two different model types in order to eliminate the disadvantages of the other with their advantages. The hypertrophy pathway is extremely complex and its effects on the whole body are even more complex. With our research, we wanted to promote a deeper understanding of this special area of sports science, which in my opinion succeeded, but I feel that new questions were raised. I mentioned earlier that we continued our acute occlusion study, now as a chronic occlusion training program, the results of which were surprising. The

reader may think that multiple sampling would have been necessary to obtain even more accurate results, but its application is no longer ethically beneficial.

The alternative hypertrophy methods mentioned above offer exceptional opportunities for the investigation of both extreme and general adaptation manifestations. The "intention" of the genes was examined with the PCR tests, while the "fulfillment" of the will of the genes was examined with the Western blot procedure. The examination of miRNAs discovered during the last decade shows us an even more complete picture of the processes taking place in the body.

11. List of own publications

Announcements related to the dissertation

Gombos, Zoltan, Erika Koltai, Ferenc Torma, Peter Bakonyi, Attila Kolonics, Dora Aczel, Tamas Ditroi, Peter Nagy, Takuji Kawamura, és Zsolt Radak. 2021. „Hypertrophy of Rat Skeletal Muscle Is Associated with Increased SIRT1/Akt/MTOR/S6 and Suppressed Sestrin2/SIRT3/FOXO1 Levels". International Journal of Molecular Sciences 22 (14): 7588. https://doi.org/10.3390/ijms22147588.

Torma, Ferenc, Zoltan Gombos, Marcell Fridvalszki, Gergely Langmar, Zsofia Tarcza, Bela Merkely, Hisashi Naito, és mtsai 2021. „Blood Flow Restriction in Human Skeletal Muscle during Rest Periods after High-Load Resistance Training down-Regulates MiR-206 and Induces Pax7". Journal of Sport and Health Science 10 (4): 470–77. https://doi.org/10.1016/j.jshs.2019.08.004.

Announcements not related to the dissertation

Babszky, Gergely, Ferenc Torma, Dora Aczel, Peter Bakonyi, Zoltan Gombos, Janos Feher, Dóra Szabó, és mtsai. 2021. „COVID-19 Infection Alters the Microbiome: Elite Athletes and Sedentary Patients Have Similar Bacterial Flora". Genes 12 (10): 1577. https://doi.org/10.3390/genes12101577.

Torma, Ferenc, Peter Bakonyi, Zsolt Regdon, Zoltan Gombos, Matyas Jokai, Gergely Babszki, Marcell Fridvalszki, és mtsai. 2021. „Blood Flow Restriction during the Resting Periods of High-Intensity Resistance Training Does Not Alter Performance but Decreases MIR-1 and MIR-133A Levels in Human Skeletal Muscle". Sports Medicine and Health Science 3 (1): 40–45. https://doi.org/10.1016/j.smhs.2021.02.002.

Torma, Ferenc, Zoltan Gombos, Matyas Jokai, Istvan Berkes, Masaki Takeda, Tatsuya Mimura, Zsolt Radak, és Ferenc Gyori. 2020. „The Roles of MicroRNA in Redox Metabolism and Exercise-Mediated Adaptation". Journal of Sport and Health Science 9 (5): 405–14. https://doi.org/10.1016/j.jshs.2020.03.004.

Torma, Ferenc, Zoltan Gombos, Matyas Jokai, Masaki Takeda, Tatsuya Mimura, és Zsolt Radak. 2019. „High Intensity Interval Training and Molecular Adaptive Response of Skeletal

Muscle". Sports Medicine and Health Science 1 (1): 24–32.
https://doi.org/10.1016/j.smhs.2019.08.003.