The Role of the PGC-1α in Intramuscular Lipid Metabolism

Abstract of PhD Thesis

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1. Introduction

The worldwide epidemic of metabolic diseases, such as obesity and type 2 diabetes, highlights the importance of understanding the role of skeletal muscle and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) in regulating energy metabolism. Skeletal muscle is an adaptive and important tissue for movement and regulation of metabolism, which acts as the largest endocrine organ that regulates metabolic processes and health. This adaptability is mainly controlled by the PGC-1α gene, which is highly expressed in bioenergetically active tissues such as skeletal muscle. The functions of PGC-1α are regulated by complex networks involving enzymes such as AMPK (adenosine 5'-monophosphate-activated protein kinase) and sirtuin 1 (SIRT1), which act upstream of PGC-1α signaling to activate its downstream pathways These enzymes help skeletal muscle cells respond to energy demands by modulating metabolic processes.

Intramuscular triglyceride (IMTG), lipid droplets stored within skeletal muscle fibers, is an important energy source, especially during endurance exercises. Adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) are key lipases that regulate IMTG breakdown via the lipolysis process and utilization. The distribution and accumulation of IMTGs significantly affect muscle function, metabolic health, and the development of metabolic disorders.

It has been shown that PGC-1α improves skeletal muscle's capacity to generate energy from IMTG by increasing the number of mitochondria as well as mitochondrial energy-producing enzymes and affecting lipid breakdown enzymes. For instance, the activation of the SIRT1/PGC-1α/Fibronectin Type III Domain-Containing Protein 5 (FNDC5) axis can enhance lipolysis by stimulating lipolytic enzymes.

Although PGC-1α has been known for its role in mitochondrial biogenesis, in this study we aimed to explore its specific role in regulating intramuscular triglyceride metabolism. We used 10-month-old male mice, including both wild-type and transgenic C57BL/6-Tg, to study how the overexpression of PGC-1α in muscle affects IMTG metabolism in response to exercise. This research involved 40 middle-aged mice, comparing both sedentary and exercise-trained groups over ten weeks of intervention. Specifically, this study was designed to evaluate the distinct and simultaneous effects of PGC-1α and endurance exercise on IMTG metabolism. We evaluated mitochondrial function, mitochondrial health markers, lipid metabolism enzymes, and signaling

molecules to understand the mechanisms of mitochondrial change and lipid breakdown in skeletal muscle.

This study aimed to improve our understanding of skeletal muscle responses to exercise and PGC-1α overexpression. These findings have the potential to develop optimal exercise techniques for improving metabolic health, as well as the development of treatments for metabolic diseases including obesity, insulin resistance, and diabetes.

2.Objectives

This research was designed to explore the mechanisms of muscle-specific PGC-1α overexpression and exercise-related adaptation dependent on IMTG metabolism. The primary goal was to determine if the changes in IMTG metabolism are mainly caused by PGC-1α or affected by other exercise-related factors. PGC-1α is recognized for its established functions in mitochondrial biogenesis and fatty acid oxidation; however, its specific influence on IMTG metabolism is not fully understood.

2.1 Hypotheses

Based on the literature review and identified research gaps, we developed the following hypotheses:

Hypothesis 1: PGC-1α overexpression in skeletal muscle and endurance exercise training improve mitochondrial health and function markers, leading to enhanced endurance performance.

Hypothesis 2: PGC-1α overexpression in skeletal muscle and endurance exercise training will improve triglyceride breakdown markers, leading to increased reliance on fat as an energy source in the quadriceps muscle tissue of mice.

Hypothesis 3: PGC-1α overexpression in skeletal muscle and endurance exercise training modulate the expression of key enzymes involved in mitochondrial dynamics, contributing to improved metabolic flexibility and exercise adaptation in the quadriceps muscle tissue of mice.

Hypothesis 4: PGC-1α overexpression in skeletal muscle and endurance exercise training change key signaling pathways, such as the AMPK-α and sirtuin pathways, in the quadriceps muscle tissue of mice.

2.2 Relevance of the Research

The results of this study would improve our understanding of the molecular mechanisms underlying the adaptations of skeletal muscles induced by exercise. Understanding the complex interplay between exercise, metabolism, and genetics will allow us to determine how PGC-1α regulates mitochondrial activity and intramuscular lipid metabolism. The findings have significant implications for developing therapeutic strategies for metabolic diseases such as obesity, type 2 diabetes, and cardiovascular disease.

3. Material and Methods

3.1 Animals: Housing, Study Groups, and Ethical Approval

In this study, we used 40 C57BL/6-Tg (Ckm-Ppargc1a)31Brsp/J mice. These mice, a transgenic strain, express the PGC-1α gene more specifically in their skeletal muscle, under the control of the mouse muscle creatine kinase promoter. This genetic modification leads to a change in muscle fiber composition, resulting in more type II oxidative phenotype than normal wild-type mice. Such transgenic models are particularly valuable for investigating skeletal muscle physiology, exercise adaptations, oxidative capacity, and metabolic homeostasis. We selected male mice to reduce hormonal changes that could alter muscle metabolism, as female hormonal cycles might add additional considerations. All mice were ten months old, which is considered middle age for this strain. Middle age in C57BL/6-Tg mice ranges from 8 to 15 months, which is consistent with The Jackson Laboratory's definition of middle age as 10–14 months for these mice. To ensure the validity of our results, we randomly distributed the mice into four experimental groups while maintaining a balance in age to minimize the risk of selection bias by forming groups with similar baseline characteristics.

We compared physiological parameters in the presence and absence of PGC-1α overexpression using two sedentary control groups. The first group consisted of eleven mice with normal wild-type levels of PGC-1α (wt-C), while the second group included eleven mice with genetically induced overexpression of PGC-1α (PGC-1α-C).

Two additional groups of mice underwent an exercise program to investigate the effects of exercise. The PGC-1α exercise group (PGC-1α-Ex) comprised 9 mice with high levels of PGC-1α

who participated in the exercise program. Similarly, the wild-type exercise group (wt-Ex) included nine mice with normal levels of PGC-1α who also underwent the exercise program.

We kept all the animals in the animal house located in the Research Center for Molecular Exercise Science, Hungarian University of Sports Science in Budapest, which houses all of its mice in an environmentally controlled animal facility. Particular regulations for temperature, humidity, light cycle (12 hours of light and dark), and ventilation were followed by this facility to guarantee the best possible circumstances for the care of the animals. We freely provided the mice with standard laboratory chow and water, ensuring their constant access to sustenance.

The National Animal Research Ethical Committee of Hungary approved ethical standards and criteria that were followed throughout all the experimental processes. This study, approved by the ethical committee under approval number PE/EA/62-2/2021, guarantees that all animal handling, manipulations, and data collection procedures were carried out humanely and with the highest regard for the animal's welfare.

3.2 Training Protocol

After familiarizing the mice in the training groups with the treadmill, we used a fatigue endurance test to practically measure their maximal running capacity, a key metric for calculating the animals' maximal running capacity as it is explained previously. According to the data obtained from the endurance test, we started the training at 60 percent of the animals' maximal running capacity and gradually increased the exercise intensity each week.

Training started at 60% of each mouse's maximum running capacity. The training continued for 10 weeks, with five days of 30-minute training sessions per week

3.3 Western Blot Protocol

3.3.1 Tissue collection and homogenization:

Following accepted ethical standards, 48 hours after the last training session, we humanely put the mice to sleep to remove their quadriceps tissue. We removed the quadriceps muscles, a key muscle region involved in running and submerged them in liquid nitrogen to quickly freeze the tissue and maintain its metabolic integrity. We then kept the frozen muscle samples at -80 °C to ensure their quality and stability for further molecular analysis.

3.3.2 Tissue homogenization

We used an accurate weight scale to separate 50–80 grams of quadriceps muscle in the same way for all samples. We then homogenized them using a lysis buffer containing 137 mM NaCl, 20 mM Tris-HCl pH 8.0, 1% Nonidet P-40, 10% glycerol, plus protease SIGMAFASTTM Protease Inhibitor Tablets (S8820-20TAB) and PhosSTOPTM phosphatase (4906845001) inhibitor tablets to prevent degradation. We placed the muscle tissues in this lysis buffer within an ice container and then homogenized them using an Ultra Turrax homogenizer (IKA, Staufen im Breisgau, Germany).

3.3.3 Protein extraction and concentration determination

We used the Bradford assay to check the protein content of the tissues and make sure that all of them had the same amount of protein for loading into polyacrylamide (SDS-PAGE) gels for western blots. To do this, we used Bio-Rad's protein assay dye reagent concentrate (#5000006) that had been diluted four times and the BCA protein concentration assay as a standard (Sigma A3059) to find out how much protein was in the mixed samples. We calibrated the SDS-PAGE Western blot loading amount by balancing the protein content of different samples based on the measured protein concentration.

3.3.4 Electrophoresis and Detecting Bands

After having proper samples for loading in 8–12% SDS-PAGE, we loaded 4–8 µl of samples inside the gels to perform electrophoresis. Following the completion of the electrophoresis process, we transferred the proteins from the gels to PVDF membranes using the Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (1,703,940). Then, by using a 5% Milk Tris-buffered saline-Tween-20 (TBST) solution, we blocked the membranes for one hour at room temperature. The incubation step of the target-specific antibody took place at night in the 4 °C fridge. The primary antibodies used for incubation include: HSL rabbit (1:1000, cell signaling 18381S), ATGL rabbit (1:1000, cell signaling 2439 S), mTOR rabbit (1:1000, cell signaling 2983S), Sirt3 rabbit (1:1000, cell signaling 2627), PCYT2 rabbit (1:1000, Thermofisher PA5-90,366), AMPK-α rabbit (1:1000, cell signaling 2532), eNOS mouse (1:1000, abcam ab76198), Phospho-eNOS (Ser1177) rabbit (1:1000, cell signaling (9571), Sirt1 mouse (1:1000, abcam, ab110304), LONP1 mouse (1:3000, Proteintech − 66,043–1-Ig), SDHA rabbit (1:3000, SantaCruz—sc-98253), CS rabbit (1:1000, abcam,ab96600), PGC-1α rabbit (1:3000, Novusbio, NBP1-04676), Fis1 rabbit (1:1000,

SantaCruz, sc98900), Mfn1 rabbit (1:1000, SantaCruz, sc50330), FNDC5 rabbit (1:1000, abcam, ab174833), GPR41 rabbit (1:1000, Thermofisher, PA5-75,521), GPR43 rabbit (1:500, Thermofisher, PA5-111,780), nNOS mouse (1:1000, BD Transduction Laboratories, 610,309), NAMPT/Visfatin rabbit (1:1000,abcam, ab45890), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mouse (1:40,000;Sigma-Aldrich, 9001–50-7), α-Tubulin mouse (1:20,000, Sigma-Aldrich,T6199). On the following day, the incubated membranes went through a washing process with TBST buffer 4 times 8 min at room temperature, then we performed the second antibody incubation process in room temperature for one hour according to the host species of primary antibody. The anti-rabbit and anti-mouse IgG HRP-conjugated secondary antibodies (Jackson Immunoresearch) were used, which were diluted 1:10,000 in a 5% milk TBST solution. Next, we washed the membranes four times for 8 minutes at room temperature using TBST buffer. The washed membranes were incubated for one minute in chemiluminescent reagent SuperSignal™ West Pico PLUS Chemiluminescent Substrate REF-34580. We used the AZURE 400 Visible Fluorescent Imager (AZI400-01) for detecting specific protein bands on the membranes by applying Wide Dynamic Range cumulative imaging mode. After obtaining band spots on the membranes, we used ImageJ software version 1.53t to quantify them. The values were normalized by housekeeping proteins, which in our study were GAPDH and α-Tubulin. Also, the phosphorylation ratio was calculated by using the same PVDF membrane after stripping the phosphorylated form of the proteins and incubating the same membrane in the total protein antibodies.

3.4 Statistical analysis

We used GraphPad Prism version 9.1.0 software to perform statistical analysis and analyze experimental data. Our reported data includes mean values with either a standard deviation (SD) or a standard error of the mean (SEM). The significance level for all analyses was set at $p<0.05$.

Since our experimental design comprised four distinct groups, we implemented a two-way analysis of variance (ANOVA) as the primary statistical method. This technique allowed us to assess the significance of differences among groups and experimental contexts.

We continued our analysis by applying Fisher's LSD post hoc test to detect significant differences among different groups of the study. We selected this post hoc test due to its effectiveness in identifying comparisons, which enables a thorough exploration of the intricate variances in our

experimental data. These statistical methods strengthened the validity of our data analysis and made our statistical results more significant and reliable.

4. Results

4.1 The effects of PGC-1α overexpression on running distance

The comparison of running distances to exhaustion showed a statistically significant difference between the Wild Type (wt-Ex) and PGC-1α overexpressed (PGC-1α-Ex) groups, both at baseline and after exercise training. This important finding suggests that overexpressing PGC-1α in skeletal muscle improves endurance ability and probably increases resistance to exhaustion during exercise. Significantly, even before exercise training, PGC-1α overexpression was associated with longer running distances, highlighting its innate function in controlling muscle performance. These findings suggest that PGC-1α may facilitate modifications such as higher mitochondrial content and increased fatty acid oxidation capacity, that help delay fatigue during endurance exercise. The advantages of these findings might be important in developing strategies for preventing muscle fatigue, improving endurance performance, or controlling muscle weakness in different diseases related to muscle function.

4.2 The effects of PGC-1α overexpression and exercise training on mitochondrial and oxidative stress marker levels

Comparing wild-type mice with those overexpressing PGC-1α, western blot analysis showed overexpression of PGC-1α in mice's skeletal muscle resulted in significant differences in protein expression levels linked to their mitochondrial function. The experiment's results clearly showed significant differences in the protein levels of the two groups. Increased PGC-1α, FNDC5, LONP1, CS, SDHA, and Mfn1 levels were linked to PGC-1α overexpression, but SIRT3 levels were lower. According to these results, PGC-1α overexpression may have an impact on several metabolic pathways, such as those involved in mitochondrial biogenesis (CS, SDHA), mitochondrial dynamics (Mfn1), and possibly cellular signaling (FNDC5, SIRT3). Most importantly, the effects of exercise training on protein levels vary based on whether PGC-1α overexpression was applied or not. The exercise training regimen increased the Wt-Ex group's PGC-1α and Fis1, the proteins that play the main role in the mitochondrial fission process. On the other hand, PGC-1α, SIRT3, and Fis1 all showed increases in response to exercise training in the PGC-1α-Ex group. These

findings demonstrate the intricate relationship between skeletal muscle changes brought on by exercise and PGC-1α overexpression.

4.3 The effects of PGC-1α overexpression and exercise training on metabolic and adaptive capacity-related markers

Comparing wild-type mice with those overexpressing PGC-1α, western blot analysis showed overexpression of PGC-1α in mice skeletal muscle resulted in higher levels of AMPK-a, a key marker for cellular energy. Furthermore, these animals had elevated levels of mTOR, a protein synthesis and cell proliferation factor, indicating a possible connection between PGC-1α and mTOR signaling pathways. Furthermore, the PGC-1α overexpressed animals showed elevated levels of SIRT1 protein, a crucial regulator of cellular processes, highlighting the complex interactions between these regulatory elements. The endothelial phosphorylated NOS (peNOS) to eNOS ratio significantly increased in transgenic animals, suggesting a possible effect of PGC-1α on the vascularization of the skeletal muscle. On the other hand, animals that overexpressed PGC-1α showed decreased levels of neuronal NOS (nNOS), suggesting that PGC-1α may have different regulatory functions in controlling subtypes of NOS in skeletal muscle. Moreover, analyzing the effects of exercise training showed that both the PGC1-Ex and wt-Ex groups had a significant increase in SIRT1 protein content. This finding contributes to the possibility of a synergistic effect between exercise and PGC-1α, which could account for the observed improvements in metabolic flexibility and muscle performance.

4.4 The effects of PGC-1α overexpression and exercise training on lipid metabolism markers

Comparing wild-type mice with those overexpressing PGC-1α, western blot analysis showed that overexpression of PGC-1α in mice skeletal muscle resulted in higher levels of GPR41 and PCYT2, suggesting that PGC-1α may have regulatory functions in the expression of these genes. In the case of the effects of exercise training on lipase levels, In the case of the effects of physical training, this intervention increased the levels of HSL and ATGL key lipases involved in triglyceride breakdown among both groups (wt-Ex and PGC-1α-Ex).

5. Conclusion

In conclusion, our findings provide strong evidence that PGC-1α plays a key role in skeletal muscle adaptation to exercise by modulating mitochondrial dynamics and key signaling pathways. Additionally, PGC-1α and exercise independently influence lipid breakdown. The novel findings regarding the interaction between PGC-1α and GPR41, PCYT2 are important, suggesting potential new pathways for therapeutic intervention.

In this section, we conclude that our hypothesis evaluation supports the role of PGC-1α in modulating mitochondrial dynamics and lipid metabolism while also highlighting the independent effects of exercise on these processes.

Hypothesis 1: PGC-1α overexpression in skeletal muscle and endurance exercise training improve mitochondrial health and function markers, leading to enhanced endurance performance.

Accepted: Our results validate our first hypothesis. This is shown by PGC-1α overexpressing mice which can run longer distances and are less likely to get tired, which is in line with PGC-1α's known role in supporting mitochondrial function and oxidative metabolism. The results also indicate that exercise training positively affected performance, as evidenced by post-test assessments and an increase in PGC-1α levels in wild-type mice that underwent the exercise training program. Additionally, higher levels of important mitochondrial markers like CS, SDHA, LONP1, and PGC-1α, which indicate a greater capacity for mitochondrial biogenesis, health, and energy production marker adaptation. This further supports our hypothesis that both PGC-1α overexpression and exercise training enhance endurance performance.

Hypothesis 2: PGC-1α overexpression in skeletal muscle and endurance exercise training will improve triglyceride breakdown markers, leading to increased reliance on fat as an energy source in the quadriceps muscle tissue of mice.

Partially accepted. The hypothesis that exercise training increases triglyceride breakdown enzymes is confirmed, as exercise training led to an increase in lipolytic enzymes. In our study, overexpression of PGC-1α alone did not significantly affect the enzymes HSL and ATGL. However, animals with PGC-1α overexpression that underwent exercise showed higher enzyme levels than those exercising wild-type animals. Furthermore, to understand the broader metabolic effects related to triglyceride breakdown, we measured the expressions of the fatty acid receptors

GPR41 and GPR43 in the skeletal muscles of mice. Previous research has shown that these receptors affect the activation of AMPK-α, an enzyme that increases PGC-1α activity resulting in elevated number and improved function of mitochondria. Our findings indicated that GPR41 expression increased in response to PGC-1α overexpression, whereas GPR43 expression remained unchanged. Interestingly, our results showed increased expression of PCYT2, an important lipid biosynthesis regulator for muscle health and aging, which may play a role in muscle adaptation to metabolic changes induced by PGC-1α overexpression. Notably, this increase in PCYT2 expression was not observed with exercise alone. This adds a new dimension to understanding how lipid metabolism is regulated in skeletal muscle.

Hypothesis 3: PGC-1α overexpression in skeletal muscle and endurance exercise training modulate the expression of key enzymes involved in mitochondrial dynamics, contributing to improved metabolic flexibility and exercise adaptation in the quadriceps muscle tissue of mice.

Accepted: Our results confirm this hypothesis, showing that PGC-1α overexpression increases Mfn1 levels, enhancing improved structural integrity and fused mitochondria, while endurance exercise significantly increases Fis1 levels, a marker of the process of breaking down into new mitochondria. The findings from the PGC-1α-Ex group suggest that PGC-1α and exercise are linked in regulating mitochondrial dynamics, validating our third hypothesis that both interventions influence the proteins involved in this process.

Hypothesis 4: PGC-1α overexpression in skeletal muscle and endurance exercise training change key signaling pathways, such as the AMPK-α and sirtuin pathways, in the quadriceps muscle tissue of mice.

Partially Acceptable: Our results showed that endurance training alone did not affect SIRT3 levels in wild-type mice. However, in PGC-1α overexpressing animals, endurance training effectively normalized the previously reduced SIRT3. This indicates that the effect of exercise on SIRT3 is likely dependent on the presence of PGC-1α, as evidenced by the normalization of SIRT3 levels in PGC-1α overexpressing animals. In contrast, SIRT1 levels increased in response to both interventions. PGC-1α binds to the promoter region of the SIRT1 gene and activates its transcription, thereby increasing the production of the SIRT1 protein. This interaction between PGC-1α and SIRT1 forms a positive feedback loop that regulates cellular energy homeostasis and mitochondrial function. However, regarding AMPK-α as an upstream

regulator of PGC-1α, we found that PGC-1α can coactivate AMPK-α, leading to increased AMPK-α activity. PGC-1α can reduce the production of reactive oxygen species and upregulate antioxidant enzymes. This is supported by our results, which show increased phosphorylation of the eNOS enzyme. Thus, we conclude that PGC-1α helps to preserve the eNOS protein and maintain its activity. Unexpectedly, nNOS levels decreased in response to PGC-1α overexpression and remained unchanged by exercise. These findings partially support our hypothesis that PGC-1α overexpression and endurance exercise have distinct and independent effects on key signaling pathways. However, the combined effects of PGC-1α overexpression and endurance exercise did not consistently produce synergistic outcomes, highlighting the complex nature of their interactions as observed in the study. To better understand how PGC-1α and exercise training affect skeletal muscle adaptation, more research is required to explore the specific mechanisms.

6. List of own publications

Publications related to the dissertation

Mozaffaritabar S, Koltai E, Zhou L, Bori Z, Kolonics A, Kujach S, Gu Y, Koike A, Boros A, Radák Z. PGC-1α activation boosts exercise-dependent cellular response in the skeletal muscle. J Physiol Biochem. 2024 May;80(2):329-335. doi: 10.1007/s13105-024-01006-1. Epub 2024 Jan 23. PMID: 38261146; PMCID: PMC11074013.

Lei Z, Mozaffaritabar S, Kawamura T, Koike A, Kolonics A, Kéringer J, Pinho RA, Sun J, Shangguan R, Radák Z. The effects of long-term lactate and high-intensity interval training (HIIT) on brain neuroplasticity of aged mice. Heliyon. 2024 Jan 10;10(2):e24421. doi: 10.1016/j.heliyon.2024.e24421. PMID: 38293399; PMCID: PMC10826720.

Publications not related to the dissertation

Zsolt Radák , Dóra Aczél , Iván Fejes , Mozaffaritabar Soroosh , Gabor Pavlik , Zsolt Komka , László Balogh , Zsofia Babszki , Gergely Babszki , Erika Koltai , Kristen M. McGreevy , Gordevicius Juozas , Steve Horvath , Csaba Kerepesi

Slowed epigenetic aging in Olympic champions compared to non-champions

GEROSCIENCE: OFFICIAL JOURNAL OF THE AMERICAN AGING ASSOCIATION (AGE) 2024 Paper: 10.1007/s11357-024-01440-5 PMID: 39601999

Zhou L, Mozaffaritabar S, Kolonics A, Kawamura T, Koike A, Kéringer J, Gu Y, Karabanov R, Radák Z. Long-term iron supplementation combined with vitamin B6 enhances maximal oxygen uptake and promotes skeletal muscle-specific mitochondrial biogenesis in rats. Front Nutr. 2024 Jan 15;10:1335187. doi: 10.3389/fnut.2023.1335187. PMID: 38288063; PMCID: PMC10823527.

Radak Z, Pan L, Zhou L, Mozaffaritabar S, Gu Y, A Pinho R, Zheng X, Ba X, Boldogh I. Epigenetic and "redoxogenetic" adaptation to physical exercise. Free Radic Biol Med. 2024 Jan;210:65-74. doi: 10.1016/j.freeradbiomed.2023.11.005. Epub 2023 Nov 17. PMID: 37977212.

Bakonyi P, Kolonics A, Aczel D, Zhou L, Mozaffaritabar S, Molnár K, László L, Kutasi B, Tanisawa K, Park J, Gu Y, Pinho RA, Radak Z. Voluntary exercise does not increase gastrointestinal motility but increases spatial memory, intestinal eNOS, Akt levels, and *Bifidobacteria* abundance in the microbiome. Front Physiol. 2023 Aug 16;14:1173636. doi: 10.3389/fphys.2023.1173636. PMID: 37664431; PMCID: PMC10468588.