# Investigation of pharmacological compounds to increase skeletal muscle glucose uptake *in vitro* and *in vivo*

Ph.D. Thesis

Zoltán Márton Köhler


Supervisor: *Anikó Keller-Pintér, M.D., Ph.D.*

Doctoral School of Multidisciplinary Medical Sciences

Department of Biochemistry

Albert Szent-Györgyi Medical School

UNIVERSITY OF SZEGED

SZEGED

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# LIST OF PUBLICATIONS

1. The complete list of papers directly related to the subject of the thesis:

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2. Gönczi, Mónika, Andrea Csemer, László Szabó, Mónika Sztretye, János Fodor, Krisztina Pocsai, Kálmán Szenthe, Anikó Keller-Pintér, **Zoltán Márton Köhler**, Péter Nánási, Norbert Szentandrássy, Balázs Pál, and László Csernoch (2022). Astaxanthin Exerts Anabolic Effects via Pleiotropic Modulation of the Excitable Tissue. *International Journal of Molecular Sciences 23*(2):917. https://doi.org/10.3390/ijms23020917 [IF: 5.6] D1

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3. Szepesi, Ágnes, László Bakacsy, Henrietta Kovács, Árpád Szilágyi, and **Zoltán Márton Köhler** (2022). Inhibiting Copper Amine Oxidase Using L-Aminoguanidine Induces Cultivar and Age-Dependent Alterations of Polyamine Catabolism in Tomato Seedlings. *Agriculture (Switzerland) 12*(2):274. https://doi.org/10.3390/agriculture12020274 [IF: 3.6] Q2
4. Becskeházi, Eszter, Marietta Margaréta Korsós, Eleonóra Gál, László Tiszlavicz, Zsófia Hoyk, Mária A. Deli, **Zoltán Márton Köhler**, Anikó Keller-Pintér, Attila Horváth, Kata Csekő, Zsuzsanna Helyes, Péter Hegyi, and Viktória Venglovecz (2021). Inhibition of NHE-1 Increases Smoke-Induced Proliferative Activity of Barrett's Esophageal Cell Line. *International Journal of Molecular Sciences 2021, Vol. 22, Page 10581 22*(19):10581. https://doi.org/10.3390/ijms221910581 [IF: 6.208] D1

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# 1. INTRODUCTION

## 1.1. Muscle homeostasis

Skeletal muscles, constituting up to 40% of body weight, play crucial roles in breathing, movement, posture, and metabolism. They possess remarkable self-renewal ability through satellite cells, ensuring structure and function restoration post damage. Nutrient availability is vital for this process, with glucose influencing the activated satellite cells, namely myoblast differentiation; deficiency can hinder skeletal muscle fiber regeneration and contribute to conditions like sarcopenia. Age-related changes also impact glucose uptake and metabolic pathways, affecting muscle health and function.

## 1.2. Signal transduction pathways regulating skeletal muscle glucose uptake

In a healthy state, skeletal muscle plays the greatest role in normalizing elevated blood glucose levels through the action of the glucose transporter type 4 (GLUT4). It is the only one glucose transporter in the body that is not found in the plasma membrane in the basal state and is insulin-dependent. In myoblasts, the presence of both GLUT4 and GLUT1 is important, however, after differentiation, the significance of GLUT1 becomes negligible in myotubes.

The translocation of GLUT4 to the membrane can be initiated by the binding of insulin to the insulin receptor. The receptor activates phosphoinositide 3-kinase (PI3K) through insulin receptor substrate 1 (IRS1) phosphorylation. On one hand, PI3K is able to provide the way by initiating the rearrangement of the actin skeleton through the Ras-related C3 botulinum toxin substrate 1 (Rac1)/p21-activated kinase 1 (PAK1)/Cofilin pathway. On the other hand, it can activate the motile elements through the 3-phosphoinositide-dependent protein kinase 1 (PDK1)/Akt/Akt substrate of 160 kDa (AS160; TBC1D4) pathway. In the absence of phosphorylation, AS160 can keep the Rab molecules responsible for GLUT4 trafficking in an inactive form, thereby retaining GLUT4 in intracellular vesicles.

Alternatively, GLUT4 translocation can also be initiated in an insulin-independent way by AMP-activated protein kinase (AMPK), which can also regulate AS160. AMPK, acting as an energy sensor, is capable of sensing the AMP/ATP ratio and getting activated in response to high levels of AMP. AMPK, together with sirtuin 1 (Sirt1), is able to regulate peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), which is a key transcription factor in many biological processes, such as mitochondrial biogenesis and glucose/fatty acid metabolism. And peroxisome proliferator-activated receptor-γ (PPARγ), which is directly responsible for the transcription of GLUT4, is also under the control of PGC-1α. Akt and

AMPK have a common, although oppositely regulated element, mammalian target of rapamycin (mTOR), which is responsible for glucose/fatty acid/amino acid metabolism, thereby impacting complex processes like cell growth and survival.

### 1.3. Impairment of GLUT4 translocation and diabetes mellitus

GLUT4 is the limiting element of skeletal muscle glucose uptake, and impaired GLUT4 translocation and reduced GLUT4 amount are the main causes of insulin resistance. Insulin resistance and inadequate insulin secretion are the underlying causes of type 2 diabetes mellitus (T2DM), also known as non-insulin-dependent diabetes mellitus (DM). T2DM accounts for 90% of all DM. The chronic hyperglycemic state characteristic of diabetes is capable of inducing oxidative stress in multiple tissues, leading to various complications in the long term. Typical manifestations include stroke, cardiovascular diseases, peripheral neuropathy, retinopathy, and diabetic nephropathy. Furthermore, as the treatment of diabetes mellitus advances, new complications have been identified, such as cancer, infections, functional and cognitive impairments, liver diseases, and emotional disorders. Despite the severity of the situation, the prevention and cure of the disease have not yet achieved a breakthrough, as the number of diagnosed patients continues to increase every year, reaching 536.6 million at present, and based on the trend, it is projected to reach 634 million patients by 2030. The situation is made worse by the fact that diabetics are probably not even diagnosed. Despite all this, there are few therapeutic options that would help glucose uptake at the level of signaling. Metformin is the only drug still in use today in this respect that activates AMPK and enhances glucose uptake by downregulating the mitochondrial function.

### 1.4. Bone morphogenetic proteins (BMPs) and the regulation of glucose uptake

Bone morphogenetic proteins (BMPs) belong to the superfamily of transforming growth factor β (TGF-β) proteins and, in addition to regulating the formation of bones and cartilage as originally described, they also play a role in various other biological processes, such as adipogenesis, obesity, and diabetes. BMPs are secreted proteins, and their effects on cells are mediated through their heterodimeric receptors, leading to the phosphorylation and activation of multiple Smad molecules.

BMPs can be involved in the regulation of glucose uptake. BMP7 can increase glucose uptake in both muscle and adipose tissue via the PDK1/PI3K/Akt pathway and GLUT4 translocation. Consistent with this, its amount decreased in the serum of T2DM patients, while the amount of BMP2 increased. However, BMP2 together with BMP6 have an insulin-

sensitizing effect on adipocytes, increased the translocation of GLUT4 and increase the amount of GLUT4 via PPARγ. BMP6 alone is capable to reduce serum lipid and glucose levels in ob/ob mice after 6 days. BMP4 expression is increased in response to hyperglycemia and free fatty acids. Moreover, in humans, BMP4 levels correlate with adipocyte size, obesity, and insulin sensitivity. Unfortunately, the complex effects of BMPs are still not fully understood, and there is a lack of comprehensive studies investigating their combined actions.

### 1.5. Tilorone dihydrochloride

An antiviral drug known and used since the 1970s, tilorone dihydrochloride (2,7-bis[2-(diethylamino)ethoxy]-9-fluorenone dihydrochloride; hereafter: tilorone), has been described to enhance the transcription of BMP2 and BMP7 in lung epithelial cells. Tilorone is able to increase Smad1/5/8 phosphorylation through BMPs, thereby inhibiting the loss of muscle mass and improving survival in mice suffering from muscle cachexia.

Tilorone is an antiviral agent marketed in several countries and indicated against viral infections such as influenza, acute respiratory viral infection, viral hepatitis, and viral encephalitis. Furthermore, it is also effective against many viruses that are prevalent today, such as herpes simplex virus, West Nile virus, Ebola virus, and human coronaviruses including MERS-CoV and SARS-CoV-2.

### 1.6. The relationship between reactive oxygen species and glucose uptake

Reactive oxygen and nitrogen species (ROS/RNS) generated during metabolism are essential when balanced by antioxidants. Disrupted redox balance leads to oxidative stress, causing many diseases like T2DM. ROS include superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical ($^{\bullet}OH$), which can damage biomembranes, proteins, enzymes, and nucleic acids, leading to cell damage and senescence. Most ROS form in mitochondria during ATP production, with factors like high glucose triggering mitochondrial dysfunction, inflammation, and apoptosis. Impaired mitochondrial function can lead to lipid accumulation, insulin resistance, and reduced glucose uptake. ROS-induced serine phosphorylation inhibits tyrosine phosphorylation of IRS by insulin, so insulin signaling itself, Akt and AS160 affected GLUT4 translocation, and ROS reduce the amount of GLUT4 and ultimately glucose uptake. Peroxynitrite inhibits insulin action by reducing IRS1 phosphorylation on tyrosine and suppressing Akt activation. Restoring and maintaining redox balance is vital to prevent these harmful effects.

### 1.7. Antioxidant defense and astaxanthin

The human body is equipped with a number of antioxidants that are used to counteract the effects of oxidants. They can be divided into two categories: enzymatic and non-enzymatic. Astaxanthin is a xanthophyll carotenoid that also belongs to non-enzymatic antioxidants. It can be synthesized by many, mainly marine algae, and it is present in various animals through the food chain, so it can also be found in human foods.

Astaxanthin, like other carotenoids, is lipid-soluble, able to fit into the lipid bilayer and trap free radicals both in the membrane and on the surface of the membrane. Astaxanthin is a more potent non-enzymatic antioxidant than Vitamin C, β-carotene, and α-tocopherol. It is capable of significantly reducing elevated ROS levels induced by high glucose, TNFα, or palmitic acid. And astaxanthin able to reduce level of blood sugar and HbA1c, as well as the value of TG and cholesterol in T2DM patients.

## 2. AIMS OF THE THESIS

The effects of individual BMPs on glucose uptake and metabolism have been investigated earlier. This has allowed us to understand that certain members of BMP family influence glucose uptake through GLUT4 expression and its translocation. Furthermore, serum BMP levels are related to insulin sensitivity and T2DM. However, the combined effects of BMPs are still unknown. It has been proven that ROS reduces the amount of GLUT4 and impairs GLUT4 translocation, leading to insulin resistance in the long term. However, the changes caused by the elimination of ROS with antioxidants are still incomplete in terms of regulating GLUT4 translocation. In this way, we set ourselves the goal of expanding our knowledge in relation to the following questions:

- Is tilorone really able to simultaneously increase the levels of several BMPs and activate the BMP signaling pathway in myoblasts?
- Does tilorone treatment have an effect on the proteins controlling GLUT4 translocation in myoblasts and myotubes?
- Can tilorone treatment increase the levels of GLUT1 and GLUT4, as well as glucose uptake in myoblasts?
- Is tilorone treatment able to sensitize myotubes to insulin?

- What is the effect of tilorone treatment on the amount and function of mitochondria of myoblasts?
- Does systemic tilorone treatment affect tissue glucose uptake in mice?
- Does astaxanthin feeding have an influence on the molecules that control glucose uptake in the skeletal muscle of mice?

# 3. MATERIALS AND METHODS

## 3.1. Cell culture, differentiation and treatment

C2C12 mouse myoblast cells were maintained in 80% high-glucose Dulbecco's modified Eagle's medium (DMEM) and 20% fetal bovine serum (FBS), which even contained 50 μg/ml gentamicin. Proliferating cells were treated with 20 or 35 nM 2,7-bis[2-(diethylamino)ethoxy]-9-fluorenone dihydrochloride (tilorone dihydrochloride) for 40 h.

For differentiation, in the medium of 80-90% confluent cells, FBS was replaced by 2% horse serum and the cells were grown into myotubes for 5 days. Differentiated myotubes were treated with 20 nM tilorone (2 or 5 h), and in some studies with 100 nM insulin for 10 minutes.

## 3.2. Experimental animals

12-week-old male C57BL/6J mice were included for tilorone vaccinations. For the 4-week astaxanthin feeding experiment, 3-month-old male C57BL/6 mice were used. Astaxanthin-containing AstaReal A1010 was added to the normal rodent pellet at 4 g/kg. The animal experiments were approved by the Ethical Committee for Animal Research, University of Debrecen, Hungary (1/2017/DEBÁB, 3-1/2019/DEMÁB). Laboratory animals were kept and treated in compliance with all applicable sections of the Hungarian Laws and animal welfare directions and regulations of the European Union.

## 3.3. Qantitative real-time (qRT)-PCR

For qRT-PCR, total RNA was isolated from myoblasts and reverse transcribed. The SybrGreen reactions were performed with qPCRBIO SyGreen Mix Lo-ROX mix according to the manufacturer's instructions, using the following primers: [BMP2: NM_007553.3; BMP4: NM_007554.3; BMP7: NM_007557.3; GDF5 (BMP14): NM_008109.3; Smad4: NM_008540.2; Slc2a1 (GLUT1): NM_011400.3; Slc2a4 (GLUT4): NM_001359114.1; HPRT: NM_013556.2; RPL27: NM_011289.3]. Individual threshold cycle ($C_t$) values were

normalized to the mean $C_t$ values of *MmHprt* and *MmRpl27* as internal control genes. Relative gene expression levels are presented as $2^{-\Delta\Delta Ct}$.

### 3.4. Gel electrophoresis and immunoblotting

C2C12 cells were lysed in RIPA buffer supplemented with 1 mM NaF and protease inhibitors. Biceps femoris, and pectoralis muscles of control and astaxanthin-fed mice were homogenized in buffer containing 50 mM of Tris-HCl pH 7.6, 100 mM of NaCl, 10 mM of EDTA, 1 mM of NaF, 1 mM of $Na_3VO_4$, and protease inhibitor cocktail.

The cell lysates were centrifuged for 5 min, while the muscle homogenates were centrifuged for 10 minutes at 16,000×g at 4 °C to eliminate cellular debris. Protein concentration in the samples was determined using a BCA protein assay kit and equal amounts of proteins were separated on SDS-polyacrylamide gel and transferred onto Protran membranes. After blocking with skimmed milk, the membranes were incubated overnight with the primer mouse/rabbit primary antibodys. Following incubation with the appropriate horse-radish peroxidase-conjugated anti-IgG secondary antibodies, peroxidase activity was visualized by enhanced chemiluminescent method and the chemiluminescence signal was recorded using X-ray. Quantification of signal intensity was carried out by Quantity One software.

### 3.5. Fluorescent staining and microscopy

For fluorescent staining, the samples were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.2% Triton X100, and blocked with 2% BSA in PBS. Rabbit anti-GLUT4 primary antibody followed by Alexa Fluor 488-conjugated secondary antibody was used to visualize GLUT4. To label the mitochondria, the cells were incubated with MitoTracker™ Deep Red FM under normal conditions and then fixed with 4% paraformaldehyde for 15 min at room temperature. The nuclei were counterstained with Hoechst 33258.

Wide-field fluorescence images were obtained using a Nikon Eclipse Ni-U fluorescence microscope with a 100× objective (Nikon CFI Plan Apo DM Lambda 100× Oil, NA = 1.45). Images were normalized to their background, then signal intensity was quantified and plotted normalized to control. The evaluation and conversion of the images to a heat maps were performed with ImageJ software.

Leica DMi1 phase-contrast microscope with a 10× objective (Leica Hi Plan 10×, NA = 0.28) was used to generate representative images to show the morphology of undifferentiated (myoblast) and differentiated (myotube) C2C12 cells.

### 3.6. *In vitro* $^{18}$F-fluoro-2-deoxyglucose ($^{18}$FDG) uptake measurement

To monitor the uptake of the radioactive glucose analog $^{18}$FDG by myoblast cells, C2C12 cells were cultured for 24-26 hours. Then cells were incubated in the presence of 10 µCi (0.37 MBq) of $^{18}$FDG in 1 mL DMEM containing 1mM glucose and 20% FBS for 1 h at 37°C. Finally, the cells were harvested, resuspended and the radioactivity of the suspension was measured with Packard Cobra-II Auto Gamma Counter device.

Tilorone treatment was performed at concentrations of 20 and 35 nM for 40 hours. Tilorone was also present during $^{18}$FDG incubation. To test the role of GLUT1 in glucose uptake, tilorone-treated cultures were incubated with a GLUT1 inhibitor, BAY-876 10 nM for 2 h.

To measure the $^{18}$FDG uptake of differentiated myotubes, the cells were plated in a proliferation medium for 24 hours, then at 90% confluence the 20% FBS content of the medium was replaced with 2% horse serum and the cells were differentiated into myotubes for 5 days. On the 5th day on differentiation, cell cultures were treated with 20 nM tilorone for 2 or 5 h and 100 nm insulin for 10 min. $^{18}$FDG uptake was measured as described previously.

### 3.7. Assessment of mitochondrial oxygen consumption ($O_2$ flux) using high-resolution respirometry

To examine the respiration of different experimental groups, myoblasts ($3 \times 10^6$ cells) were suspended in 2 mL MiR05 respiration medium (pH 7.1; oxygen solubility factor 0.92) and gently pipetted into the oxygraph chambers.

In intact cells, after stable routine respiration, ATP synthase was inhibited with oligomycin for blocking mitochondrial ATP synthesis. Maximal capacity of the electron transport system (ETS) was achieved with stepwise titration of protonophore. Following complex I inhibition, the electron transport system-independent respiration (or residual oxygen consumption) was determined in the presence of complex III inhibitor, antimycin A. DatLab software was used for display, respirometry data acquisition, and analysis.

### 3.8. Animal treatment and *in vivo* imaging

C57BL/6J mice were injected intraperitoneally with a dose of 25 mg tilorone/kg body weight. The treatment was repeated 3 days after the first injection, and the positron emission tomography / computed tomography (PET/CT) scans were performed before the first vaccination and after the second vaccination.

Mice were anaesthetized with 3% Forane and were injected with 10.2±0.9 MBq of $^{18}$FDG in 100 μL saline before treatment and after the second tilorone treatment. 50 min after radiotracer injection, whole-body PET/CT scans were performed in isoflurane anaesthesia using the preclinical *nanoScan PET/MRI* 1T device. After the image reconstruction and PET image analysis, the standardized uptake value (SUV) was calculated by the following formula: SUV= [ROI activity (MBq/mL)]/[injected activity (MBq)/animal weight (g)].

### 3.9. Statistical analysis

Statistical evaluations were performed via Student's t-test or one-way analysis of variance (ANOVA) followed by Dunnett's or Sidak post-hoc test. GraphPad Prism 8.0 was used for graphing and statistical analyses. The data are expressed as the mean + standard errors of the means (SEM) or the median with the 25th and 75th percentiles are plotted on box plots. A $p < 0.05$ was considered significantly different.

## 4. RESULTS

### 4.1. Tilorone increases BMP signaling in myoblasts

First, we investigated the effect of tilorone on the transcription of BMPs in C2C12 myoblasts using the treatment time and concentration described in the literature. qRT-PCR revealed that mRNA levels of BMP2, BMP4, BMP7, and BMP14 were elevated by both (20 and 35 nM) tilorone concentrations in myoblasts; however, BMP14 expression was only significantly increased by the higher concentration. Activation of the BMP signaling pathway was indicated by an increase in Smad4 mRNA level and an increase in the phosphorylation of Smad1/5/8(Ser463/Ser456/Ser467).

### 4.2. Effect of tilorone on signaling molecules regulating GLUT4 translocation

We investigated how the increased BMP transcription and activated BMP signaling effected by tilorone affects the regulatory elements of glucose uptake. Tilorone increased the

phospho-Akt2(Ser474)/Akt2 and phospho-AMPK(Thr172)/AMPK ratios, and these changes were significant for 35 nM tilorone. The full amount of AS160 expression was decreased by both tilorone concentrations, and the phospho-AS160(Thr642)/AS160 ratio was increased.

To test the short time effect of tilorone, we used myotubes, which differentiation were confirmed by the increased expression of desmin. In myotubes, 20 nM tilorone increased the phospho-AS160(Thr642)/AS160 ratio after either 2 and 5 h treatment.

### 4.3. Tilorone increases the expression of GLUTs and glucose uptake of C2C12 cells

We examined the intracellular expression of GLUT4 in tilorone-treated myoblasts using immunofluorescent staining and wide-field fluorescence microscopy. Quantification of the whole GLUT4 fluorescence intensity of the cells revealed an increase in their GLUT4 content after tilorone treatment. Subsequently, qRT-PCR was conducted to examine the mRNA levels of Slc2a4 (GLUT4) and the myoblast-specific Slc2a1 (GLUT1). It was found that treatment with various concentrations of tilorone resulted in increased levels of both Slc2a1 and Slc2a4 transcripts. Examining the effect of tilorone treatment with the Western blot technique, and while the level of GLUT1 increased tendentiously, GLUT4 increased at both concentrations. Using the Western blot technique, we examined the expression of PPARγ, which is a critical regulator of GLUT4 expression, we detected an increase in response to the two concentrations of tilorone we used.

After the increased activity of the elements regulating GLUT4 translocation and the increased amount of GLUT4, we wondered whether the glucose uptake by the cells was consistent with all of this. Our results revealed a 1.5-fold increase in $^{18}$FDG uptake after 20 nM tilorone treatment, and this uptake was further amplified by the higher concentration. To separate GLUT1- and GLUT4-linked glucose uptake, we used a GLUT1-specific inhibitor, BAY-876, which reduced cellular $^{18}$FDG uptake.

### 4.4. Tilorone increases $^{18}$FDG uptake of myotubes and enhances insulin effect

In myotubes, by increasing the phosphorylation of Akt2(Ser474), we verified the effect of insulin and examined the insulin-sensitizing effect of tilorone. During this investigation, it was observed that prior tilorone treatment further enhanced the phospho-Akt2(Ser474)/Akt2 ratio after insulin administration.

After that, the $^{18}$FDG uptake of the myotubes was examined and we observed increased $^{18}$FDG uptake of myotubes after tilorone treatment (5 h). Insulin increased the $^{18}$FDG uptake

of control and tilorone-treated (2 or 5 h) groups. Notably, tilorone administration (5 h) further boosted the insulin-mediated $^{18}$FDG uptake of myotubes.

### 4.5. Effects of tilorone treatment on mitochondrial function in myoblasts

A high-resolution respirometry was used to examine the effect of increased glucose uptake on mitochondrial respiratory chain and indirectly the production of oxidative ATP. Compared to the control group, treatment with 20 or 35 nM tilorone resulted in a significant decrease in routine respiration in intact myoblasts. Additionally, tilorone also reduced ATP-linked respiration.

Due to the functional changes, we examined the total amount of PGC-1α, which regulates the biogenesis of mitochondria, and found no change in its protein level. Therefore, we labeled the mitochondria of the cells with MitoTracker™ Deep Red FM and individually examined the total mitochondrial content of myoblasts, where we also did not find any differences in response to tilorone treatment.

### 4.6. Tilorone administration increases $^{18}$FDG uptake *in vivo*

Based on the increased glucose uptake observed in skeletal muscle myoblasts and myotubes *in vitro*, we proceeded to investigate the effect of systemic tilorone administration on *in vivo* $^{18}$FDG uptake using PET/CT imaging. By performing quantitative analysis of the $^{18}$FDG-PET images, we observed a significant increase in the average SUV (Standardized Uptake Value) of skeletal muscle, adipose tissue, and liver following tilorone treatment, indicating enhanced radiotracer uptake in these tissues.

### 4.7. Astaxanthin feeding influences the activation of signaling molecules affecting skeletal muscle metabolism

Due to their size, skeletal muscles are responsible for a significant part of the metabolism of the entire body. Therefore, we aimed to investigate whether the dietary application of a potent antioxidant, astaxanthin, has an effect on the proteins regulating skeletal muscle metabolism. The effects of long-term astaxanthin administration on representative muscles known to be involved in force production such as the hindlimb (biceps femoris muscle) or forelimb (pectoral muscle) muscles of the mice were studied.

In both examined muscles, the phosphorylation of mTOR(Ser2448) increased as a result of astaxanthin feeding. However, the total amount of mTOR also showed an increase, thus the phospho-mTOR(Ser2448)/mTOR ratio did not change significantly after astaxanthin

treatment. Although the Akt2 and the phospho-Akt2(Ser474)/Akt2 ratio did not change significantly in the studied samples, phospho-AS160(Thr642)/AS160 ratio increased in both biceps femoris and pectoral muscle samples. The expression of PGC-1α showed only a slight increase in the biceps femoris and pectoralis muscle samples following astaxanthin treatment.

## 5. DISCUSSION AND CONCLUSIONS

In skeletal muscle cells, whether they are myoblasts, myotubes, or skeletal muscle tissues, glucose uptake is a complex mechanism in which the regulation of GLUT4 translocation plays a central role. If the sensing of initiating signals and the signaling pathway are impaired, GLUT4 translocation is hindered, leading to a lack of glucose uptake. As a result, persistently high blood sugar levels develop, which can cause a multitude of severe complications throughout the body. To avoid this, it is important to gain a deeper understanding of the regulatory mechanisms of GLUT4 translocation and discover new signaling processes.

The new findings of this thesis can be summarized as follows:

- Tilorone treatment increased the expression of BMP2, BMP4, BMP7 and BMP14; furthermore, activated the BMP signaling pathway in C2C12 cells.
- The activation of regulatory elements of GLUT4 translocation, such as phospho-Akt2(Ser474)/Akt2 and phospho-AS160(Thr642)/AS160 ratios, increased as a result of tilorone treatment in myoblasts, thereby favouring GLUT4 translocation.
- We observed that as a result of tilorone treatment, the amount of PPARγ increased and the levels of GLUT1 and GLUT4 increased in myoblasts.
- Following tilorone treatment, the phospho-AS160(Thr642)/AS160 ratio increased in myotubes. Moreover, upon insulin stimulation, the increase in phospho-Akt2(Ser474)/Akt2 ratio further elevated in tilorone treated samples.
- After tilorone treatment, uptake of the glucose analog $^{18}$FDG increased in both myoblasts and myotubes. Tilorone treatment further enhanced insulin-induced $^{18}$FDG uptake in myotubes.
- The significantly increased glucose uptake did not result in increased ATP generation by mitochondrial respiration; both basal and ATP-linked respiration decreased in myoblasts after tilorone treatment, contributing to the induction of AMPK, increasing the ratio of phospho-AMPK(Thr172)/AMPK.

- In the *in vivo* experiments, the systemic administration of tilorone resulted in increased $^{18}$FDG uptake of skeletal muscle, liver, and adipose tissue in C57BL/6J mice.
- Astaxanthin feeding did not change the ratio of the insulin-dependent regulator of GLUT4 translocation, phospho-Akt2(Ser474)/Akt2, in either the biceps femoris or the pectoralis muscle of C57BL/6 mice, while that of phospho-AS160(Thr642)/AS160 ratio increased.
- Phosphorylation of mTOR(Ser2448) increased as a result of astaxanthin feeding in either the biceps femoris and the pectoralis muscle of C57BL/6 mice, but the total mTOR amount also increased, so the phospho-mTOR(Ser2448)/mTOR ratio did not change.

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