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Investigation of the Role of Smoothelin-like Protein 1 in Skeletal Muscle During Hyperthyroidism

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Introduction

Overactive Thyroid: Hyperthyroidism

Hyperthyroidism occurs when the thyroid gland produces excessive amounts of thyroid hormones. The three most common causes of hyperthyroidism are: autoimmune Grave's disease, toxic multinodular goiter and toxic thyroid adenoma. General symptoms of hyperthyro idism include soft, warm skin, heat intolerance, excessive sweating, increased metabolism and weight loss despite an increased appetite. Palpitations, tachycardia and the development of insulin resistance are common. Patients are usually nervous, irritable and have tremors. Proximal muscle weakness occurs in 50% of the cases.

The Effects of Hyperthyroidism on Skeletal Muscle

Hyperthyroidism affects all tissues and organs of the human body, especially the skeletal muscle. Skeletal muscle comprises approximately 40% of total body weight and is essential for maintaining posture, producing movement, breathing and speech. It also plays a fundamental role in processes such as energy production or glucose and lipid homeostasis. Consequently, any changes affecting the skeletal muscle have a significant impact on the whole body.

Skeletal muscle consists of multinucleated, striated muscle fibers. There are 4 main types of mammalian skeletal muscle fibers: 1, 2a, 2x and 2b. Slow twitch type 1 fibers are resistant to fatigue, have a smaller diameter and express myosin heavy chain (MyHC) 1 and sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) 2a, which is associated with a poorly developed sarcoplasmic reticulum. They are rich in mitochondria and have an extensive capillary network. In addition, their sarcoplasm is low in glycogen and high in triglyceride and myoglobin; the latter gives the characteristic red color of these fibers. They have a predominant aerobic metabolism. In contrast, their glycolytic capacity and phosphocreatine levels are low. Fast twitch type 2 fibers have a larger diameter, express SERCA1a, have a well-developed sarcoplasmic reticulum and are surrounded by a less extent capillary network than type 1 fibers. Their energy needs are covered by anaerobic glycolysis, which is an energetically unfavorable but rapid way of ATP production. The glucose required for this process is derived from the extracellular space or from intracellular glycogen stores, the end product is lactate. White-colored type 2b fibers expressing MyHC 2b are capable of high force exertion due to their very high glycogen and phosphocreatine content, associated with a small number of mitochondria; however, they fatigue rapidly under intense stress. In contrast, the pink type 2a fibers expressing MyHC 2a have both high oxidative and

glycolytic capacity due to high mitochondria, phosphocreatine and myoglobin content. Therefore, they are less fatigable but exert less force compared to type 2b fibers. The most recently discovered type 2x fibers expressing MyHC 2x are transitional in their properties between type 2a and 2b fibers. Although there are muscles composed almost exclusively of one type of fiber, such as the soleus of rodents (type 1), most mammalian skeletal muscles have a combination of type 1 and type 2 fibers, in which the quality and quantity of expressed MyHC proteins are specific to the muscle.

In hyperthyroidism, excess of thyroid hormone greatly alters the fiber composition of the muscle by regulating the expression of MyHCs: depending on the initial fiber type, MyHC 1 becomes 2a, 2a becomes 2x and 2x becomes 2b. The increase in the number of fast type fibers leads to more contraction and relaxation and, consequently, an increase in the rate of energy production and use. In addition, thyroid hormones stimulate the expression and/or activity of the main energy-consuming proteins e.g. SERCA and Na^+/K^+-ATPase (NKA) in muscle cells. In order to maintain all of these, more ATP is needed, but due to the increase in lactate secretion and thermogenesis, the efficiency of ATP production decreases. The increased glucose demand is provided by increased breakdown of stored glycogen and by stimulating extracellular glucose uptake. Nevertheless, a reduced responsiveness of skeletal muscle to normal insulin concentrations has been observed during hyperthyroidism, which is manifested by a decrease in insulin-mediated glucose uptake of skeletal muscle.

Although numerous genetic and physiological factors are involved in the development and exacerbation of insulin resistance, studies involving human subjects point to altered activity of insulin receptor substrate (IRS) proteins as a common mechanism underlying insulin resistance. In addition to Tyr side chains, IRS1 can also be phosphorylated on Ser/Thr side chains, the latter stimulating or inhibiting IRS1 activity in a position-dependent manner. For example, IRS1 Ser phosphorylation by protein kinase B (PKB)/Akt protects against Tyr phosphatases, keeping IRS1 active through a positive feedback mechanism. Insulin, however, also activates other Ser/Thr kinases e.g. extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), protein kinase C (PKC) and mammalian target of rapamycin (mTOR) that induce phosphorylation of specific Ser side chains that inhibit IRS1 function by a negative feedback mechanism, resulting in the abolition of insulin action. The balance between positive Tyr/Ser and negative Ser phosphorylation plays an important role in the regulation of IRS1 activity and normal insulin signaling. This balance can be disturbed by various stressors (e.g. diseases) and molecules (e.g.

hormones, amino acids, cytokines, fatty acids), resulting in a predominance of negative Ser phosphorylation and the development of insulin resistance.

Smoothelin-like Protein 1 (SMTNL1) as a Key Regulator of Skeletal Muscle Plasticity

One potential regulator of skeletal muscle insulin resistance is SMTNL1, which is remarkable for its ability to regulate the adaptation of skeletal muscle to different stresses (e.g. exercise, pregnancy) in a sex-dependent manner. Deletion of SMTNL1 induces changes in skeletal muscle fiber composition: western blot analysis of plantaris muscle from pregnant SMTNL1 knockout (KO) female mice revealed a significant increase in the expression of MyHC 2b proteins and a decrease in the expression of MyHC 2a proteins, resulting in a shift in the ratio of 2b/2a fibers. According to the respiratory quotient of pregnant and non-pregnant SMTNL1 KO mice, non-pregnant SMTNL1 KO mice mainly utilize carbohydrates as fuel over fatty acids and proteins. In addition, it was also observed that the physical activity of SMTNL1 KO mice was significantly lower compared to that of wild type (WT) mice, indicating a decrease in metabolic efficiency. The intraperitoneal glucose tolerance test showed that SMTNL1 KO mice displayed reduced glucose tolerance. Reduced glucose tolerance leads to the development of insulin resistance, which is presumably a consequence of reduced glucose transporter 4 (GLUT4) and IRS1 expression due to SMTNL1 KO.

Relationship between SMTNL1 Protein and Myosin Phosphatase (MP) Enzyme

During skeletal muscle development, MP is regulated by SMTNL1. MP is a heterotrimeric protein composed of a 37 kDa catalytic subunit (PP1c), a 110-130 kDa regulatory subunit (MYPT) and a 20 kDa subunit (M20) of an unknown function. Three isoforms of the MYPT regulatory subunit are known (MYPT1, MYPT2, MYPT3), which are responsible for directing the catalytic subunit to different substrates and regulating the activity of the holoenzyme. Several mechanisms have been identified to regulate MP holoenzyme activity. One of them is the phosphorylation of the MYPT regulatory subunit by Ser/Thr kinases, which results in activation or inhibition of the holoenzyme. For example, phosphorylation of the Thr696 of the MYPT1/2 subunit is inhibitory. MP enzyme activity is also regulated by protein-protein interactions. One of these interaction partners is the SMTNL1 protein, which binds directly to the MYPT1 subunit in the cytosol in its dephosphorylated state. Phosphorylation of the Ser301 side chain causes SMTNL1 to dissociate from MYPT1 and translocate to the nucleus, releasing the MP enzyme

from inhibition. The significance of the regulation of MP by SMTNL1 in skeletal muscle function remains unclear. Inhibition or silencing of the regulatory subunit of MP and pharmacological inhibition of its catalytic subunit significantly slow down cell migration. Since migration plays an important role in skeletal muscle development and because of the migration of skeletal muscle progenitor cells in hyperthyroidism is less studied, I continued my research in this direction investigating the potential regulatory role of SMTNL1 in these processes. In my experiments, I worked with the immortalized C2C12 mouse myoblast cell line, which was established from the leg muscle of normal adult female C3H mice. It is a widely used model to study skeletal muscle differentiation and metabolism *in vitro*.

Myogenesis involves the proliferation, migration and differentiation of progenitor cells, which is essential for the proper development of skeletal muscle. Among the factors that control myogenesis, thyroid hormones and the proteins involved in their mechanism of action, such as deioidinase 2 (DIO2) and thyroid hormone receptor α (TRα), are of paramount importance. During the proliferative phase of C2C12 myoblast cells, TRα inhibits the expression of transcription factors, for example myoblast determination protein 1 (MyoD1) and myogenin that stimulate the progression of differentiation, independently of the presence of triiodothyronine (T3). DIO2 expression increases in differentiating myoblasts, which is associated with an increase in intracellular T3 concentration. Elevated T3 levels result in the repression of transcription factors that inhibit differentiation, such as AP-1, thereby increasing the expression of MyoD1 and myogenin. During the terminal differentiation of myocytes into myotubes, among others the presence of T3, myogenin and MYF4 is essential for the expression of proteins that determine the contractile and metabolic properties of skeletal muscle fibers, e.g. MyHC or SERCA. Last but not least, it has been shown that the phosphorylation state of myosin also plays an important role in the formation of myotubes, therefore myosin light chain kinase (MLCK) and MP are inevitable in this process. When the phosphorylation of myosin is altered, myoblast fusion is allowed, but smaller myotubes are formed.

Aims

Both hyperthyroidism and the deletion of SMTNL1 have a significant impact on skeletal muscle structure and function, thereby influencing the energy homeostasis of the whole body. The studies reported in this thesis are the first to elucidate the relationship between hyperthyroidism accompanied by insulin resistance and SMTNL1 protein and their effects on skeletal muscle remodeling, with particular emphasis on the expression and activity of myogenic, contractile and metabolic proteins. Based on these observations, the main objectives of the experimental work were the following:

1) Investigation of the role of SMTNL1 protein in the regulation of skeletal muscle metabolism during hyperthyroidism:
   - expression analysis of endogenous SMTNL1 protein and marker proteins of different types of fibers in human euthyroid and hyperthyroid skeletal muscle biopsies
   - investigation of the effect of hyperthyroidism on the expression of genes and their protein products involved in various metabolic pathways in human euthyroid and hyperthyroid skeletal muscle samples
   - investigation of the effect of T3 and SMTNL1 protein on the expression and activity of proteins involved in insulin signaling and glucose metabolism in a cellular model system
   - investigation of the role of T3 and SMTNL1 proteins in determining the metabolic phenotype of C2C12 cells

2) Investigation of the role of SMTNL1 protein in the regulation of C2C12 cell homeostasis and T3-dependent myogenesis:
   - investigation of the effect of T3 and SMTNL1 on myoblast cell migration and differentiation
   - investigation of the role of T3 treatment and differentiation on the expression of the regulatory and catalytic subunits of MP enzyme
   - examination of the regulatory effects of T3 and SMTNL1 protein on the expression and activity of MP enzyme and its substrates in myoblasts and myotubes

Materials and Methods

Maintenance of Cell Culture

C2C12 myoblasts at passage number 6-9 were grown in DMEM supplemented with 1000 mg/L glucose, 2 mM L-glutamine, 10% (v/v) foetal bovine serum and phenol red (growth medium) at 37 °C and 5% CO_2.

Transient Transfection of C2C12 Cells with Recombinant SMTNL1 Protein

Plasmid DNA that expresses N-terminal Flag-tagged recombinant SMTNL1 protein (NT-FT-SMTNL1) was added to serum-free DMEM/ GeneJuice transfection reagent mixture at a ratio of 3:1 and was incubated for 15 minutes. Then, the entire volume of the transfection mixture was added drop-wise to the cells in complete growth medium. Finally, cells were seeded in to collagen-coated tissue culture plates and were incubated at 37 °C overnight (O/N). Transfection efficiency was confirmed by western blot analysis using anti-Flag antibody.

Differentiation and Treatment of C2C12 Cells

Myogenic differentiation was induced at 90% confluency by replacing the growth medium with phenol red-free DMEM supplemented with 1000 mg/L glucose, 2 mM L-glutamine and 2% (v/v) horse serum (HS) (differentiation medium) and cells were incubated for 6 days. Differentiation medium was also completed with 10 nM T3 from the first day or from the beginning of the 4th day. In case of control, 1 nM T3 was added to the cells. Differentiation was monitored by western blot analysis using antibodies specific for myogenic markers. Treatment with a PKA agonist (8-bromoadenosine-3'-5'-cyclic monophosphate; 8BrcAMP) was conducted on NT-FT-SMTNL1-transfected myotubes at a final concentration of 10 µM for 24 hours.

Cell Lysis

Cells were harvested in ice-cold radioimmunoprecipitation assay (RIPA) buffer [25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% sodium deoxycholate, 0.1% sodium-dodecyl-sulphate (SDS), 1% Triton-X 100, 1 mM phenylmethylsulphonyl (PMSF), 10x protease inhibitor cocktail (PIC), 50x phosphatase inhibitor cocktail (PPIC), 1 µM microcystin (MC-LR)] on ice. Cell suspensions were sonicated for 30 seconds with a 10% pulse and were centrifuged for 10 minutes at 16 000 x *g*, 4 °C. Supernatants were transferred to fresh tubes and total protein concentrations were measured by Bicinchoninic acid (BCA) assay.

AlamarBlue Cell Viability Assay

Myoblasts transfected with recombinant SMTNL1 were seeded at a density of 20 000 cells per well in collagen-coated 96-well plates. Following differentiation and a 72-hour T3 treatment of cells, alamarBlue reagent was added to the myotubes at a final concentration of 20 µM and were incubated for 4 hours at 37 °C. Fluorescence was measured at 530/590 nm.

Seahorse Analysis

Myoblasts were transfected with recombinant SMTNL1 and were seeded at a density of 10 000 cells per well in collagen-coated XF96 microplates. Following a 72-hour T3 treatment of cells, 180 µl of assay media containing 1000 mg/L glucose was added. Myoblasts were incubated for 1 hour in a CO_2-free incubator along with the sensor cartridge loaded with the following inhibitors in order of injection: 1) 50 µM etomoxir; 2) 2 µM oligomycin; 3) 4 µM FCCP; 4) 10 µM antimycin + 100 mM 2-deoxy-D-glucose. Five measurement points were taken for the baseline and after each injection. As a negative control, we used cell-free wells in the four corners of the XF96 plate. In all groups, 23 technical replicates were used. After the assay, cells were lysed in 1 N NaOH and total protein concentration was measured using BCA assay.

Immunoprecipitation

For the immunoprecipitation of recombinant SMTNL1 protein, we used Anti-Flag M2 Affinity Gel. To minimize unspecific binding, resin was blocked with 5% (w/v) BSA/1x TBS supplemented with 10x PIC at 4 °C O/N on a roller shaker. Recombinant SMTNL1-transfected C2C12 cells, treated with 10 nM T3 for 24 hours or 10 µM 8BrcAMP for 30 minutes, were lysed in ice-cold buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton-X 100, 1 mM PMSF, 10x PIC] on ice. Then cell lysates were incubated with protein A sepharose for 1 hour at 4 °C. Precleared cell lysates (200 µl) were added to the blocked resin and samples were agitated for 2 hours at 4 °C.

For the immunoprecipitation of MYPT and NKA, antibodies were coupled to rehydrated protein A sepharose resin in the presence of binding buffer (50 mM Tris, pH 7.0) at a ratio of 1:60 for 2 hours at 4 °C on a roller shaker. Bead-antibody complexes were washed with binding buffer and were blocked with 5% (w/v) BSA/1x TBS for 2 hours at 4 °C. The bead-antibody complexes were washed with 1x TBS and were incubated with the precleared C2C12 lysates at 4 °C O/N.

In case of both type of immunoprecipitation, beads were washed and supernatants were completely eliminated using a Hamilton-pipette. For the elution, 1x SDS sample buffer was added to the beads and samples were boiled at 100 °C for 5 minutes. Precipitates were subjected to western blot to confirm the interaction between the proteins of interest.

Immunofluorescence

SMTNL1-transfected myoblasts were seeded onto collagen-coated OptiPlate-96 Black plates at a density of 20 000 cells per well and were differentiated for 5 days. On days 0, 3 or 5 of differentiation, cells were fixed with 4% (w/v) paraformaldehyde/1x PBS for 15 minutes. Then, cells were permeabilized with 0.2% (v/v) Triton-X-100/1x PBS for 4 minutes. After blocking with 1% (v/v) HS/1x PBS for 1 h at 4 °C, immunofluorescence staining was carried out using anti-desmin (1:100) and anti-Flag (1:100) antibodies (green) and cells were incubated O/N. Fluorophore-conjugated secondary antibodies (1:200) and F-actin-binding peptide phalloidin (1:200) were incubated with the cells for 2 hours. Fluorescent signals were detected using a 10x air objective in an automated high-content imaging (HCS) reader.

Morphological Analysis

NT-FT-SMTNL1-transfected myoblasts were differentiated for 5 days. On days 0, 3 and 5 of differentiation, light microscopy images of developing monolayers were taken with a 10x air objective and morphology analysis was performed by ImageJ software to examine the morphological changes of myotubes caused by overexpression of SMTNL1. The average area and perimeter of 15-15 randomly selected myoblasts (day 0) and myotubes (days 3 and 5) per field were calculated in each case, from which we could determine the amount and size of mature myotubes. The total number of myotubes present in a field was also counted.

Scratch Assay

Myoblasts were suspended in serum-free medium containing 5 µL/mL of DiI, a highly lipophilic carbocyanine dye, and were incubated for 15 minutes at 37 °C. After washing with warm complete medium, myoblasts were transfected with recombinant SMTNL1 and were seeded onto collagen-coated OptiPlate-96 Black plates (20 000 cells per well). After 48 hours, cells were serum-starved for 5 hours and were uniformly scratched with a 10-microliter pipette

tip. Myoblasts were washed with 1× PBS and were cultured in the presence of 10 nM T_3 for an additional 24 hours under real-time screening with an HCS equipment using a 10× air objective.

Carbachol and Tautomycetin (TMC) Treatment

Plantaris muscle strips of 12 weeks old NMRI mice were removed and maintained in an organ bath filled with Krebs solution (118 mM NaCl; 4.75 mM KCl; 1.2 mM $MgSO_4$; 1.2 mM KH_2PO_4; 2.5 mM $CaCl_2$; 25 mM $NaHCO_2$; 11.5 mM glucose) warmed at 37 °C and gassed with 95% O_2 and 5% CO_2. The effects of PP1 inhibitor on muscle tissues were investigated by a 30-minute preincubation with 10 μM TMC followed by a 10-minute incubation with 10 μM carbachol. Tissues were lysed in modified RIPA buffer on ice. After centrifugation at 16 000 x *g*, 4 °C, total protein concentration of supernatants was measured by BCA assay and samples were subjected to western blot analysis. All procedures were approved by the University of Debrecen Faculty of Medicine Ethical Committee and are consistent with the NIH 'Guide for the Care and Use of Laboratory Animals'.

Western Blot Analysis

After cell lysis or tissue homogenization, samples were incubated with 4x SDS sample buffer for 5 minutes at 100 °C. 30 μg of protein was loaded onto 4-20% precast Criterion gels and proteins were separated at 200 V. After a 75-minute transfer at 100 V, nitrocellulose membranes were blocked in 5% (w/v) BSA/1x TBST for 1.5 hours. The blots were incubated with primary antibodies diluted in blocking solution at 4 °C, O/N. The next day, membranes were washed with 1x TBST and were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature (RT) for 1.5 hours. Immunoreactions were visualized by enhanced chemiluminescence (ECL) method.

Collection of Human Euthyroid and Hyperthyroid Skeletal Muscle Biopsies

Sternohyoid muscle biopsies from female euthyroid and hyperthyroid donors were homogenized in modified RIPA buffer [25 mM Tris (pH 7.6), 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM PMSF, 10x PIC, 50x PPIC, 1 μM MC-LR] on ice using a glass Potter-Elvehjem tissue grinder. After centrifugation at 16 000 x *g*, 4 °C, supernatants were transferred to fresh tubes and total protein concentration was measured by BCA assay. The studies involving human participants were reviewed and approved by

Regional and Institutional Ethics Committee of the University of Debrecen and were in accordance with guidelines of the European Union Council and Hungarian regulations under license number DEOEC RKEB/IKEB 3517-2011. Patients provided their written informed consent to participate in this study.

Total RNA Isolation for Microarray Analysis of Human Skeletal Muscle Biopsies

Euthyroid and hyperthyroid skeletal muscle samples were homogenized in a guanidine-isothiocyanate buffer using the TissueRuptor and were treated with proteinase K at 55 °C for 10 minutes. The supernatants were transferred to fresh tubes. Then 96-100% ethanol was added and mixed thoroughly. Traces of DNA were eliminated by DNase I treatment on the RNeasy column at 30 °C for 15 minutes. DNase I and any possible contaminants were washed away, and RNA was eluted in RNase-free water. Total RNA concentration and purity were determined using a spectrophotometer. The microarray analysis was performed using an Affymetrix Human Gene 1.0 ST Array. All raw DNA chip data is available in the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) under the following accession number: GSE178996.

Proteome Profiler Human Phospho-Kinase and Phospho-MAPK Arrays

Human euthyroid and hyperthyroid skeletal muscle biopsies were homogenized in Lysis Buffer 6 supplemented with protease and phosphatase inhibitors and samples were diluted in Array Buffer 1. For the Phospho-Kinase Array, samples were incubated with the provided membranes at 4 °C O/N and then with a cocktail of biotinylated detection antibodies at RT for 2 hours. For the Phospho-MAPK Array, samples were incubated with Detection Antibody Cocktail at RT for 1 hour. Then the prepared sample/antibody mixtures were added to the membranes that were incubated at 4 °C O/N. Finally, membranes were incubated in diluted Streptavidin-HRP and the signal was detected by ECL with the provided Chemi-Reagent Mix on X-ray films in both arrays.

Statistical Analysis

Phosphorylated proteins were normalized to non-phosphorylated protein expression, while non-phosphorylated protein expression was normalized to the loading control. Normalized data were analysed using unpaired two-tailed t-test for the comparison of two groups, and One or Two-

way ANOVA for the comparison of four or more groups followed by their corresponding *post hoc* test. Differences were considered statistically significant at $p < 0.05$.

Results and Discussion

Investigation of the Role of SMTNL1 in the Regulation of Skeletal Muscle Metabolism in Hyperthyroidism

Changes in Fiber Composition and SMTNL1 Protein Expression in Hyperthyroid Human Skeletal Muscle

Western blot analysis of sternohyoid muscle biopsies from euthyroid and hyperthyroid female patients undergoing thyroid surgery revealed that there is a significant decrease in the expression of type 1 and 2a fiber markers, MyHC I and MyHC IIa proteins. Since we did not observe any significant changes in muscle mass, it appears that hyperthyroidism led to a change in skeletal muscle fiber composition, resulting in a more glycolytic phenotype due to a decrease in oxidative fibers. Interestingly, the expression of endogenous SMTNL1 was dramatically reduced by 6-fold in hyperthyroid samples compared to euthyroid samples suggesting that SMTNL1 plays a role in the pathophysiology of hyperthyroidism in skeletal muscle.

Effect of Hyperthyroidism on the Regulation of Key Signaling Pathways in Human Skeletal Muscle

Comparison and gene ontology analysis of the global gene expression profiles of euthyroid and hyperthyroid biopsies revealed that the expression of genes involved in mTOR, AMP-activated protein kinase (AMPK), phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) signaling was most significantly altered during hyperthyroidism. The pathways are involved in biological processes such as cellular structure and organization, cell and tissue development, lipid metabolism, metabolism and energy production and their dysfunction.

To study the hyperthyroidism-induced changes in the signaling pathways identified by microarray analysis, we also examined the relative phosphorylation levels of several kinases in human skeletal muscle. The results obtained using Proteome Profiler phospho-kinase and phospho-MAPK arrays show that the phosphorylation and activity of Akt2^{S474} and mTOR^{S2448}, among others, was significantly decreased, while the activity of ERK1/2$^{T202/Y204,T185/Y187}$, AMPKα2^{T172} and JNKpan$^{T183/Y185,T221/Y223}$ was significantly increased in samples from hyperthyroid patients compared to those from euthyroid patients. Summarizing the results of the microarray and Proteome Profiler arrays, it can be concluded that hyperthyroidism has a

significant influence on skeletal muscle metabolism through alterations in the expression or activity of proteins involved in these processes.

Effect of T3 Treatment on C2C12 Cell Differentiation and Endogenous SMTNL1 Expression

To mimic adult skeletal muscle, myoblasts were differentiated into myotubes. From day 4 onwards, the expression of differentiation markers did not change significantly, indicating that differentiation was completed by day 4. To study the effect of prolonged T3 treatment on C2C12 differentiation, myoblasts were differentiated in the presence of supraphysiological (10 nM) T3 concentration for 6 days. There was no significant change in the protein expression of differentiation markers in cells treated with T3 from day 4 onwards, i.e. mature myotubes were formed by day 4, as seen in normal differentiation. In T3-treated cells, desmin expression appeared 1 day earlier compared to the control. From day 2 onwards, both markers were expressed at higher levels: desmin and MyHC expression increased significantly by 57% on day 4 and by 46% on day 6, respectively, compared to the control. This suggests that T3 promotes and accelerates the differentiation of myoblasts into myotubes.

During normal differentiation, SMTNL1 protein expression was increased gradually, but this increase was not significant. Supraphysiological T3 treatment resulted in an increase in SMTNL1 expression on day 1, which was decreased by 4-fold on day 6 compared to the control. Interestingly, SMTNL1 expression was decreased by 33% upon treatment of myotubes with 10 nM T3 for 72 hours. These results may provide evidence that long-term T3 exposure causes a decrease in SMTNL1 protein expression.

The effect of 72-hour supraphysiological T3 treatment and SMTNL1 overexpression on C2C12 cell viability was investigated using alamarBlue assay to verify that the changes caused by different treatments were not due to decreased cell number. Our experience shows that neither SMTNL1 overexpression nor supraphysiological T3 treatment affects myotube viability.

Effect of T3 Treatment and SMTNL1 Overexpression on the Expression of Thyroid Hormone Receptor Isoforms in Myotubes

After setting up the model system, we investigated the effect of SMTNL1 overexpression and supraphysiological T3 treatment on the expression of proteins responsible for T3 signaling/action. We found that there was no significant change in the expression of monocarboxylate transporter

8 (MCT8) and DIO2 under any condition, indicating that SMTNL1 protein is not involved in the regulation of thyroid hormone transport or its intracellular concentration. In contrast, the expression of TRα, an effector of thyroid hormones, was dramatically reduced by 67% and 52% by T3 treatment and SMTNL1 overexpression, respectively, which was more pronounced upon the combined treatment. These results suggest that SMTNL1 does not antagonize the effect of T3 on TRα. Interestingly, T3 treatment significantly increased TRβ expression, which was not affected by overexpression of SMTNL1 alone; however, it prevented the T3-induced elevation in TRβ expression in combination with T3.

Immunoprecipitation of recombinant SMTNL1 protein showed that there is no direct protein-protein interaction between SMTNL1 and TRs. When SMTNL1 overexpressing myotubes were treated with 8BrcAMP, we found that TRα expression was reduced by 42% as a result of the combined treatment compared to SMTNL1 overexpression alone. This suggests that SMTNL1 regulates TRα expression at the transcriptional level.

MyoD1 is a transcription factor involved in the regulation of the transcription of genes specific to type 2 fast muscle. T3 treatment alone increased the expression of MyoD1 by 55% and in combination with SMTNL1 overexpression by 49%, while SMTNL1 overexpression had no effect in myotubes. The increase in the expression of MyoD1 upon supraphysiological T3 treatment in the present model system suggests that the MyHC composition of C2C12 myotubes is also altered in favour of type 2 fibers, contributing to a more glycolytic phenotype also seen in hyperthyroidism. Since we observed a significant increase in MyoD1 expression and a concomitant decrease in SMTNL1 expression upon T3 treatment, we speculate that T3 may affect SMTNL1 expression via MyoD1. The shift in the TRα/TRβ ratio in response to supraphysiological T3 treatment and the downregulation of TRα by SMTNL1 overexpression suggest that the β isoform of TRs is the one that regulates SMTNL1 expression via MyoD1.

SMTNL1 Reduces Ser Phosphorylation of IRS1 in T3-treated Myotubes

In order to elucidate the molecular mechanism underlying insulin resistance in hyperthyroid skeletal muscle, we have studied the expression and phosphorylation of crucial members of insulin signaling, with a focus on the regulatory role of SMTNL1 in these processes. Although IRS1 expression was not significantly altered by either treatment, phosphorylation of the protein at Ser307 and Ser612 was increased by 1.6- and 1.8-fold, respectively, by T3 treatment compared to the control. Overexpression of SMTNL1 alone and in combination with T3 treatment induced

a marked decrease in the phosphorylation of IRS1 Ser307 and IRS1 Ser612. Another important component of insulin signaling is PI3K, which binds to phosphotyrosine side chains of the activated IRS1 protein. According to the microarray data, the gene expression of its 85 kDa catalytic subunit was significantly reduced in hyperthyroid human skeletal muscle samples, which was also confirmed in T3-treated myotubes at protein level. Our results provide evidence that T3 overload causes a defect in insulin signaling by increasing the inhibitory phosphorylation of IRS1 and by decreasing PI3K expression thereby contributing to a predominance of negative Ser phosphorylation and the development of insulin resistance. However, the SMTNL1 protein may be able to restore the normal insulin responsiveness of skeletal muscle by attenuating the inhibitory Ser phosphorylation of the IRS1 protein.

Regulation of IRS1 Kinases by SMTNL1 in T3-treated Myotubes

Proteome Profiler results obtained on human skeletal muscle samples show that hyperthyroidism affects the activity of kinases (mTOR, ERK1/2, JNK) potentially responsible for the increase in IRS1 negative Ser phosphorylation. To validate our results and to study the regulatory effect of SMTNL1 in C2C12 myotubes, we investigated the effect of T3 treatment and SMTNL1 protein on the expression and phosphorylation of these Ser/Thr kinases. In NT-FT-SMTNL1-transfected and T3-treated myotubes, the expression of the dephosphorylated forms of mTOR, ERK1/2 and JNK did not change significantly in response to any treatment. In contrast, phosphorylation of ERK1/2 was increased by 57% and phosphorylation of JNK was increased by 18%, whereas there were negligible changes in mTOR phosphorylation following supraphysiological T3 treatment. Remarkably, overexpression of SMTNL1 alone resulted in a significant decrease in both ERK1/2 and JNK phosphorylation and inhibited the T3-induced increase in response to the combined treatment. These results are in line with changes in Ser phosphorylation of IRS1. The exception is mTOR, where the combined treatment resulted in a significant increase in its phosphorylation compared to T3-treated cells. PKCδ may also be responsible for the stimulation of inhibitory phosphorylation of IRS1. The expression of PKCδ remained unchanged in response to T3 but it was decreased by 38 and 50% in response to SMTNL1 overexpression in the absence or presence of T3. These results may explain the enhancement of inhibitory Ser phosphorylation of IRS1 induced by supraphysiological T3 treatment and support the insulin sensitizing effect of SMTNL1. SMTNL1 is able to reduce the expression or phosphorylation of the above mentioned kinases, thereby regulating their activity.

SMTNL1 Increases GLUT4 and Decreases Hexokinase II (HK II) Expression in Myotubes

Although GLUT4 expression was increased in C2C12 myotubes in response to supraphysiological T3 treatment, its insulin-mediated translocation to the cell membrane is likely to be reduced due to inactivation of IRS1 and decreased expression of PI3K. Overexpression of SMTNL1 significantly increased GLUT4 expression, suggesting an insulin-sensitizing effect of SMTNL1 in addition to the previously discussed reduction in IRS1 inhibitory Ser phosphorylation.

In C2C12 myotubes, HK II expression was increased by 43% in response to T3 treatment, suggesting an increase in glycolysis. SMTNL1 overexpression alone and in combination with T3 treatment decreased HK II expression by 25%. AMPK also plays an important role in the regulation of skeletal muscle glucose metabolism. In hyperthyroid human skeletal muscle biopsies and supraphysiological T3-treated myotubes, we observed a significant increase in AMPK Thr172 phosphorylation. The sustained increase in AMPK phosphorylation can be explained by a decrease in the expression of protein phosphatase 2A catalytic subunit α (PP2Acα), which is responsible for the dephosphorylation of AMPK. Microarray analysis of human skeletal muscle samples revealed that the expression of the gene encoding PP2Acα was significantly decreased during hyperthyroidism, which was also confirmed at protein level in T3-treated myotubes. In contrast, overexpression of SMTNL1 did not affect the expression or phosphorylation of either AMPK or PP2Acα proteins. Based on literature data and the results presented herein, it is likely that T3 excess increases AMPK activity, which stimulates glycolysis by enhancing HK II expression. The increase in glycolysis is limited by SMTNL1 through attenuation of HK II expression.

T3 Antagonizing Effect of SMTNL1 on Extracellular Acidification Rate (ECAR) in Myoblasts

In our model system, basal ECAR and anaerobic glycolysis, indicators of lactate production, were increased by 1.4-fold upon supraphysiological T3 treatment compared to control, accompanied by a marked decrease in glycolytic reserve. In contrast, the rate of basal oxygen consumption (OCR) and aerobic glycolysis was not significantly altered upon T3 treatment. In addition to ECAR, high concentrations of T3 also enhanced fatty acid oxidation-dependent OCR, presumably due to increased AMPK activity. In contrast, overexpression of SMTNL1 not only prevented T3-induced enhancement of anaerobic glycolysis and fatty acid oxidation, but also

significantly reduced the rate of aerobic glycolysis. In the light of these data, it can be concluded that T3 overload induces an increase in ECAR and fatty acid oxidation-dependent OCR, whereas SMTNL1 antagonizes the effect of T3 through the regulation of various metabolic processes, mainly glycolysis.

Investigation of the Role of SMTNL1 in the Regulation of C2C12 Cell Homeostasis and T3-dependent Myogenesis

SMTNL1 Blocks C2C12 Myoblast Migration by Inhibiting MYPT1

Overexpression of SMTNL1 significantly impaired myoblast cell migration. This phenomenon can be explained by the inhibitory effect of SMTNL1 on MP. Our results obtained on C2C12 myoblasts showed that overexpression of SMTNL1 alone and in combination with T3 treatment significantly decreased MYPT1 expression, while phosphorylation of the inhibitory Thr696 side chain was significantly increased. In both cases, the increase in MYPT1 inhibitory phosphorylation was accompanied by a significant increase in phosphorylation of the Ser19 side chain of 20 kDa myosin light chain (MLC20), which is a long-known and well-characterized substrate of MP. Inhibition of the MP enzyme by Thr696 phosphorylation may result in slower turnover of non-muscle myosin phosphorylation, creating more stable adhesion structures that prevent cytoskeleton rearrangement and cell migration.

In contrast to SMTNL1, supraphysiological T3 treatment did not affect MYPT1 expression significantly, while its inhibitory phosphorylation and consequently cell migration was only slightly reduced. Although both short-term supraphysiological T3 treatment and SMTNL1 overexpression result in an increase in SMTNL1 protein expression, the difference in the effect of the treatments on MYPT1 expression is presumably due to the different sensitivity of the two treatments. We hypothesize that the increase in endogenous SMTNL1 expression induced by supraphysiological T3 treatment is not sufficient to affect MYPT1 expression, whereas overexpression of SMTNL1 (at physiological T3 concentration) results in an increase, which is able to effectively inhibit the expression of the MP regulatory subunit and thus myoblast migration. Supraphysiological T3 treatment induced a slight decrease in phosphorylation of MLC20, consistent with a decrease in inhibitory phosphorylation of MYPT1. Our results provide further evidence that SMTNL1 negatively regulates MYPT1 expression and activity, and that inhibition of the MP enzyme blocks cell migration. We also examined the expression of the

MYPT2 isoform in myoblasts. We found that T3 treatment alone and in combination with SMTNL1 increased MYPT2 expression, whereas SMTNL1 overexpression alone had no effect.

SMTNL1 Promotes Differentiation of C2C12 Cells

The drastic changes in myoblast migration induced by SMTNL1 have prompted us to investigate the possible regulatory role of this protein in the process of differentiation, which is also a key step in myogenesis. Under physiological T3 concentrations, SMTNL1 overexpression caused an upregulation of desmin and MyHC differentiation markers: both markers were expressed at higher levels from day 2 onwards compared to control, which resembles the effect of T3 treatment on desmin and MyHC expression. These results indicate that SMTNL1 overexpression mimics the effect of supraphysiological T3 treatment, suggesting that SMTNL1 promotes the differentiation of C2C12 myoblasts into myotubes. In addition to changes at the molecular level, we also monitored morphological alterations during differentiation. We observed that SMTNL1 overexpression induces the formation of myotubes that are significantly smaller in size but larger in number, supporting our hypothesis that SMTNL1 promotes the differentiation of C2C12 cells.

Differential Regulation of MYPT Isoforms by T3 and SMTNL1 in Myotubes

In addition to the overexpression of SMTNL1, the effect of T3 on myoblast differentiation was also studied, with particular emphasis on DIO2 and TRα. In our model system, the expression of DIO2 showed an increase under physiological conditions until day 4 (coinciding with the development of mature myotubes), and it did not change significantly from that time onwards. Supraphysiological T3 treatment gradually increased the expression of DIO2 up to day 3, but it started to decrease from day 4 onwards, suggesting a negative feedback mechanism. The expression of TRα was already detectable in myoblasts; its levels were increased during differentiation at physiological T3 concentrations, while supraphysiological T3 treatment resulted in significantly lower TRα levels on days 4, 5 and 6 (i.e. in mature myotubes) compared to control. This was consistent with the results obtained during 72 hour-T3 treatment of already differentiated myotubes.

At physiological T3 concentrations, an increase in MYPT2 expression and a non-significant decrease in MYPT1 expression were observed by the end of the 6th day, while PP1cδ expression remained unchanged throughout the entire experimental period. In myoblasts, PP1cδ is located

predominantly in complex with the MYPT1 isoform, however, in mature myotubes it is primarily MYPT2 that is involved in the assembly of the MP holoenzyme together with PP1cδ. Interestingly, supraphysiological T3 treatment increased MYPT2 expression in differentiating myoblasts, whereas it had no effect on PP1cδ and MYPT1 expression, as observed in myoblasts in the proliferative phase.

Similar results were obtained on myotubes: MYPT1 expression was not altered by T3 treatment, but SMTNL1 overexpression and combined treatment caused a significant decrease in the relative expression of MYPT1. In contrast, MYPT2 expression was increased dramatically by 56% and 54% upon T3 treatment alone and combination with SMTNL1, respectively, while SMTNL1 overexpression did not cause any change. PP1cδ expression was slightly but not significantly increased by T3 treatment and/or SMTNL1 overexpression. Summarizing the results obtained on myoblasts and myotubes, it appears that T3 and SMTNL1 selectively regulate the expression of different MYPT isoforms in both undifferentiated and differentiated cells.

Inhibitory Thr696 phosphorylation of MYPT1/2 was significantly increased by T3 and combined treatment, whereas SMTNL1 overexpression alone produced a marginally significant increase in myotubes. The inhibition of MP by T3 and/or SMTNL1 in myoblasts and myotubes indicates that in addition to the upregulation of differentiation markers, inhibition of MP enzyme may also play a role in the effects of T3 and SMTNL1 on promoting differentiation.

Effect of T3 and SMTNL1 on the Expression and Activity of an Alternative MP Substrate, NKA, in Myotubes

In C2C12 myoblasts, the coimmunoprecipitation revealed that there is an interaction between NKA and MYPT, suggesting that NKA can bind to MP through its regulatory subunit. T3 treatment alone and in combination with SMTNL1 overexpression stimulated NKA expression by 41 and 32%, respectively, whereas SMTNL1 overexpression did not changed it. The inhibitory phosphorylation of Ser23 and Ser16 of NKA was significantly increased owing to both supraphysiological T3 treatment and SMTNL1 overexpression. This is presumably due to increased inhibitory phosphorylation of the MP enzyme, which is unable to dephosphorylate NKA. This observation is also supported by treatment of mouse plantaris muscle strips with PP1 selective inhibitor (TMC), in which increased NKAα1 Ser23 phosphorylation was detected. These results suggest that both T3 and SMTNL1 are involved in the regulation of NKA phosphorylation by inhibiting MP holoenzyme through the different MYPT isoforms. When

NKA is inhibited, the membrane potential is more positive, and the cell membrane is more depolarized. Therefore, some of the voltage-gated Na^+ channels become inactive, resulting in a lower amplitude of the action potential. Consequently, less Ca^{2+} is released leading to smaller contractions and muscle weakness, which may explain the muscular symptoms of hyperthyroid patients.

Summary

During my PhD work, I have studied the regulatory role of a transcriptional cofactor, SMTNL1, in the metabolism of hyperthyroid muscle and in the myogenesis and homeostasis of C2C12 cells.

Western blot analysis of human euthyroid and hyperthyroid skeletal muscle biopsies revealed that SMTNL1 expression was dramatically decreased in response to hyperthyroidism, which was accompanied by a marked decrease in the amount of I and IIa fiber markers. We observed that the TRα/TRβ ratio was decreased, MyoD1 expression and the inhibitory Ser phosphorylation of IRS1 were significantly increased and there was a decline in PI3K expression upon supraphysiological T3 treatment, while SMTNL1 overexpression reduced the expression of TRα and IRS1 Ser phosphorylation in differentiated C2C12 cells. The elevated Ser phosphorylation of IRS1 was the result of the activation of IRS1 kinases, such as ERK1/2 and JNK, upon T3 treatment, which was supported by the Proteome Profiler data on human hyperthyroid muscle biopsies. SMTNL1 could compensate for the effects of IRS1 kinases in myotubes. In T3-treated myotubes, a decline in PP2Acα expression led to a persistent increase in AMPK phosphorylation, which enhanced glycolysis by inducing the expression of HK II. Seahorse analysis of myoblasts shed light on the increase of lactate production and fatty acid oxidation in response to supraphysiological T3, which were reversed by SMTNL1. Based on these results, we can conclude that SMTNL1 is capable of alleviating hyperthyroidism and the associated insulin resistance through its insulin sensitizing and T3 antagonizing effects.

We proved that MP consists of MYPT1 and PP1cδ in myoblasts. By inhibiting MP, SMTNL1 overexpression significantly slowed down the migration of myoblasts. Similarly to T3, SMTNL1 promoted the differentiation of myoblasts into myotubes by inducing the expression of differentiation markers. In differentiated myotubes, we found that PP1cδ is in complex with MYPT2, the activity of which was regulated by T3. The inhibition of MP by T3 or SMTNL1 caused a notable increase in the phosphorylation of NKA, which blocked the activity of the cation pump contributing to the development of muscle weakness during hyperthyroidism.

List of Publications

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List of publications related to the dissertation

1. **Major, E.**, Győry, F., Horváth, D., Keller, I., Tamás, I., Uray, K., Fülöp, P., Lontay, B.: Smoothelin-like protein 1 regulates development and metabolic transformation of skeletal muscle in hyperthyroidism.
   Front Endocrinol (Lausanne). 12, 1-17, 2021.
   DOI: http://dx.doi.org/10.3389/fendo.2021.751488
   IF: 5.555 (2020)
2. **Major, E.**, Keller, I., Horváth, D., Tamás, I., Erdődi, F., Lontay, B.: Smoothelin-like Protein 1 Regulates the Thyroid Hormone-Induced Homeostasis and Remodeling of C2C12 Cells via the Modulation of Myosin Phosphatase.
   Int. J. Mol. Sci. 22 (19), 1-18, 2021.
   DOI: http://dx.doi.org/10.3390/ijms221910293
   IF: 5.923 (2020)

List of other publications

3. Uray, K., **Major, E.**, Lontay, B.: MicroRNA Regulatory Pathways in the Control of the Actin-Myosin Cytoskeleton.
   Cells. 9 (7), 1-32, 2020.
   DOI: http://dx.doi.org/10.3390/cells9071649
   IF: 6.6
4. Horváth, D., Sipos, A., **Major, E.**, Kónya, Z., Bátori, R. K., Dedinszki, D., Szöllősi, A. G., Tamás, I., Iván, J., Kiss, A., Erdődi, F., Lontay, B.: Myosin phosphatase accelerates cutaneous wound healing by regulating migration and differentiation of epidermal keratinocytes via Akt signaling pathway in human and murine skin.
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Stem Cells Dev. 26 (23), 1724-1733, 2017.
DOI: http://dx.doi.org/10.1089/scd.2017.0035
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The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

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